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(21) International Application Number: PCT/US00/06707 (22) International Filing Date: 9 May 2000 (09.05.00) (30) Priority Data: 60/133,423 11 May 1999 (11.05.99) US (71) Applicant (for all designated States except US): ELI LILLY AND COMPANY [US/US]; Lilly Corporate Center, Indianapolis, IN 46285 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): BECKER, Gerald, Wayne [US/US]; 10815 East 121st Street, Fishers, IN 46038 (US). HALE, John, Edward [US/US]; 7644 Forest Drive, Fishers, IN 46038 (US). HEATH, William, Francis, Jr. [US/US]; 11214 Tuffton Street, Fishers, IN 46038 (US). JOHNSTONE, Edward, Marion [US/US]; 5129 East 69th Street, Indianapolis, IN 46220 (US). LITTLE, Sheila, Parks [US/US]; 4480 North Meridian Street, Indianapolis, IN 46208 (US). TU, Yuan [US/US]; 10848 Madeline Court, Fishers, IN 46038 (US). YEH, Wu-Kuang [US/US]; 720 Primrose Court, Greenwood, IN 46142 (US). YIN, Tinggui [CN/US]; 409 Mari Way, Carmel, IN 46032 (US).			(74) Agents: WILSON, Alexander et al.; Eli Lilly and Company, Lilly Corporate Center, Indianapolis, IN 46285 (US). (81) Designated States: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: AMYLOID PRECURSOR PROTEIN PROTEASE AND RELATED NUCLEIC ACID COMPOUNDS			
(57) Abstract <p>This present invention provides an amyloid precursor protein protease and related nucleic acid compounds thereof. The invention also provides compositions, expression vectors, and transfected host cells as well as assays and methods of use. The compounds of the present invention will further characterize Alzheimer's Disease and other neurodegenerative disease states.</p>			

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AMYLOID PRECURSOR PROTEIN PROTEASE AND RELATED NUCLEIC ACID COMPOUNDS

5 This invention relates to proteases which cleave amyloid precursor protein and facilitate β -amyloid peptide release, and accordingly, have utility in identifying compounds which treat or prevent Alzheimer's Disease.

 Alzheimer's disease is a degenerative brain disorder
10 characterized clinically by progressive loss of memory, cognition, reasoning, judgment, and emotional stability that gradually leads to profound mental deterioration and ultimately death. Alzheimer's disease is a very common cause of progressive mental failure (dementia) in aged humans and is
15 believed to represent the fourth most common medical cause of death in the United States. Alzheimer's disease has been observed in races and ethnic groups worldwide and presents a major present and future public health problem. The disease is currently estimated to affect about two to three
20 million individuals in the United States alone. Alzheimer's disease is at present incurable. No treatment that effectively prevents Alzheimer's disease or reverses its symptoms and course is currently known.

 The brains of individuals with Alzheimer's disease exhibit characteristic lesions termed senile (or amyloid) plaques, amyloid angiopathy (amyloid deposits in blood vessels) and neurofibrillary tangles. Large
25 numbers of these lesions, particularly amyloid plaques and neurofibrillary tangles, are generally found in several areas of the human brain important for memory and cognitive function in patients with Alzheimer's disease. Smaller numbers of these lesions in a more restrictive anatomical distribution are also found in the brains of most aged humans who do not have clinical Alzheimer's disease. Amyloid plaques and amyloid angiopathy
30 also characterize the brains of individuals with Trisomy 21 (Down's

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syndrome) and Hereditary Cerebral Hemorrhage with Amyloidosis of the Dutch-Type (HCHWA-D).

The principal chemical constituent of the amyloid plaques and vascular amyloid deposits (amyloid angiopathy), characteristic of Alzheimer's disease and the other disorders mentioned above, is an approximately 4.2 kilodalton (kD) protein of about 39-43 amino acids designated the β -amyloid peptide (this protein is also referred to in the literature as β AP, A β , A β P, A-beta, or β /A4). β -Amyloid peptide was first purified and a partial amino acid sequence was provided by Glenner, et al., Biochemical and Biophysical Research Communications, 120:885-890 (1984). The isolation procedure and the sequence data for the first 28 amino acids are described in United States Patent 4,666,829, the entire contents of which are herein incorporated by reference.

Several lines of evidence indicate that progressive cerebral deposition of β -amyloid peptide plays a seminal role in the pathogenesis of Alzheimer's disease and can precede cognitive symptoms by years or decades. See, e.g., D. Selkoe, Neuron, 6:487-498 (1991). An important line of evidence is the discovery that missense DNA mutations at amino acid 717 of the 770-amino acid isoform of amyloid precursor protein can be found in affected members, but not unaffected members, of several families with a genetically determined (familial) form of Alzheimer's disease. Goate, et al., Nature (London), 349:704-706 (1990). Genetic linkage studies have demonstrated that this mutation, as well as certain other mutations in the amyloid precursor protein gene, are the specific molecular cause of Alzheimer's disease in the affected members of such families. In addition, a mutation at amino acid 693 of the 770-amino acid isoform of amyloid precursor protein has been identified as the cause of the β -amyloid peptide deposition disease, and a change from alanine to glycine at amino acid 692 appears to cause a phenotype that resembles Alzheimer's disease in some patients. The discovery of these and other mutations in amyloid precursor protein in

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genetically based cases of Alzheimer's disease support the hypothesis that alteration of amyloid precursor protein and subsequent deposition of its β -amyloid peptide fragment can cause Alzheimer's disease.

5 Molecular biological and protein chemical analyses have shown that the β -amyloid peptide is a small fragment of a much larger precursor protein, the amyloid precursor protein (APP), that is normally produced by cells in many tissues of various animals, including humans. Knowledge of the structure of the gene encoding the amyloid precursor protein has demonstrated that the β -amyloid peptide arises as a peptide fragment that is
10 cleaved from the amyloid precursor protein by proteases.

It is presently believed that a normal (i.e., non-pathogenic) processing of the amyloid precursor protein occurs via cleavage by a putative " α -secretase" which cleaves between amino acids 16 and 17 of the β -amyloid peptide region within the protein. Kang, et al., Nature (London), 325:773-776
15 (1987). It is further believed that pathogenic processing occurs in part via an enzyme designated " β -secretase" which cleaves at the amino-terminus of the β -amyloid peptide region within the precursor protein.

The identification, isolation, and purification of the proteases involved in the processing of amyloid precursor protein would permit
20 chemical modeling of a critical event in the pathology of Alzheimer's disease and would allow the screening of compounds to determine their ability to inhibit formation of β -amyloid peptide.

Despite the progress which has been made in understanding the underlying mechanisms of Alzheimer's disease and other β -amyloid peptide-related diseases, there remains a need to develop methods and compositions
25 for treatment of the diseases. Ideally, the treatment methods would advantageously be based on drugs which are capable of inhibiting β -amyloid peptide release and/or its synthesis in vivo.

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The present invention provides an isolated protein useful as an amyloid precursor protein protease, said compound comprising the amino acid sequence

	Met	Leu	Arg	Arg	Arg	Gly	Ser	Pro	Gly	
5		1				5				
	Met	Gly	Val	His	Val	Gly	Ala	Ala	Leu	Gly
	10				15				20	
										25
10	Thr	Gly	Ala	Leu	Glu	Val	Gln	Val	Pro	Glu
					30				35	
										40
	Val	Gly	Thr	Asp	Ala	Thr	Leu	Cys	Cys	Ser
				45					50	
										55
15	Phe	Ser	Leu	Ala	Gln	Leu	Asn	Leu	Ile	Trp
				60					65	
										70
	Gln	Leu	Val	His	Ser	Phe	Ala	Glu	Gly	Gln
20										
				75					80	
										85
	Ala	Asn	Arg	Thr	Ala	Leu	Phe	Pro	Asp	Leu
				90					100	
										105
25	Ser	Leu	Arg	Leu	Gln	Arg	Val	Arg	Val	Ala
				110					115	
										120
	Cys	Phe	Val	Ser	Ile	Arg	Asp	Phe	Gly	Ser
				125					130	
										135
30	Val	Ala	Ala	Pro	Tyr	Ser	Lys	Pro	Ser	Met
				140					145	
										150
	Asp	Leu	Arg	Pro	Gly	Asp	Thr	Val	Thr	Ile
35										
				155					160	
										165

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	Gly	Tyr	Pro	Glu	Ala	Glu	Val	Phe	Trp	Gln	Asp	Gly	Gln	Gly	Val	Pro	
	170					175					180					185	
	Leu	Thr	Gly	Asn	Val	Thr	Thr	Ser	Gln	Met	Ala	Asn	Glu	Gln	Gly	Leu	
5					190					195					200		
	Phe	Asp	Val	His	Ser	Ile	Leu	Arg	Val	Val	Leu	Gly	Ala	Asn	Gly	Thr	
				205					210						215		
10	Tyr	Ser	Cys	Leu	Val	Arg	Asn	Pro	Val	Leu	Gln	Gln	Asp	Ala	His	Ser	
			220					225						230			
	Ser	Val	Thr	Ile	Thr	Pro	Gln	Arg	Ser	Pro	Thr	Gly	Ala	Val	Glu	Val	
		235					240					245					
15	Gln	Val	Pro	Glu	Asp	Pro	Val	Val	Ala	Leu	Val	Gly	Thr	Asp	Ala	Thr	
	250				255					260					265		
	Leu	Arg	Cys	Ser	Phe	Ser	Pro	Glu	Pro	Gly	Phe	Ser	Leu	Ala	Gln	Leu	
20					270					275					280		
	Asn	Leu	Ile	Trp	Gln	Leu	Thr	Asp	Thr	Lys	Gln	Leu	Val	His	Ser	Phe	
				285					290					295			
25	Thr	Glu	Gly	Arg	Asp	Gln	Gly	Ser	Ala	Tyr	Ala	Asn	Arg	Thr	Ala	Leu	
		300						305					310				
	Phe	Pro	Asp	Leu	Leu	Ala	Gln	Gly	Asn	Ala	Ser	Leu	Arg	Leu	Gln	Arg	
		315					320					325					
30	Val	Arg	Val	Ala	Asp	Glu	Gly	Ser	Phe	Thr	Cys	Phe	Val	Ser	Ile	Arg	
	330					335					340				345		
	Asp	Phe	Gly	Ser	Ala	Ala	Val	Ser	Leu	Gln	Val	Ala	Ala	Pro	Tyr	Ser	
35					350					355					360		

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Lys Pro Ser Met Thr Leu Glu Pro Asn Lys Asp Leu Arg Pro Gly Asp
 365 370 375

5 Thr Val Thr Ile Thr Cys Ser Ser Tyr Arg Gly Tyr Pro Glu Ala Glu
 380 385 390

Val Phe Trp Gln Asp Gly Gln Gly Val Pro Leu Thr Gly Asn Val Thr
 395 400 405

10 Thr Ser Gln Met Ala Asn Glu Gln Gly Leu Phe Xaa Xaa Xaa Xaa Xaa
 410 415 420 425

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 430 435 440

15 Xaa Xaa Xaa Xaa Xaa Xaa Asp Ala His Gly Ser Val Thr Ile Thr Gly
 445 450 455

Gln Pro Met Thr Phe Pro Pro Glu Ala Leu Trp Val Thr Val Gly Leu
 20 460 465 470

Ser Val Cys Leu Ile Ala Leu Leu Val Ala Leu Ala Phe Val Cys Trp
 475 480 485

25 Arg Lys Ile Lys Gln Ser Cys Glu Glu Glu Asn Ala Gly Ala Glu Asp
 490 495 500 505

Gln Asp Gly Glu Gly Glu Gly Ser Lys Thr Ala Leu Gln Pro Leu Lys
 510 515 520

30 His Ser Asp Ser Lys Glu Asp Asp Gly Gln Glu Ile Ala
 525 530 535

hereinafter referred to as SEQ ID NO:2.

35 More preferred is the amyloid precursor protein protease
 comprising the amino acid sequence

Met Leu Arg Arg Arg Gly Ser Pro Gly
1 5

35 Gly Tyr Pro Glu Ala Glu Val Phe Trp Gln Asp Gly Gln Gly Val Pro
170 175 180 185

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Leu Thr Gly Asn Val Thr Thr Ser Gln Met Ala Asn Glu Gln Gly Leu
 190 195 200
 5
 Phe Asp Val His Ser Ile Leu Arg Val Val Leu Gly Ala Asn Gly Thr
 205 210 215
 Tyr Ser Cys Leu Val Arg Asn Pro Val Leu Gln Gln Asp Ala His Ser
 10 220 225 230
 Ser Val Thr Ile Thr Pro Gln Arg Ser Pro Thr Gly Ala Val Glu Val
 235 240 245
 15 Gln Val Pro Glu Asp Pro Val Val Ala Leu Val Gly Thr Asp Ala Thr
 250 255 260 265
 Leu Arg Cys Ser Phe Ser Pro Glu Pro Gly Phe Ser Leu Ala Gln Leu
 270 275 280
 20 Asn Leu Ile Trp Gln Leu Thr Asp Thr Lys Gln Leu Val His Ser Phe
 285 290 295
 Thr Glu Gly Arg Asp Gln Gly Ser Ala Tyr Ala Asn Arg Thr Ala Leu
 25 300 305 310
 Phe Pro Asp Leu Leu Ala Gln Gly Asn Ala Ser Leu Arg Leu Gln Arg
 315 320 325
 30 Val Arg Val Ala Asp Glu Gly Ser Phe Thr Cys Phe Val Ser Ile Arg
 330 335 340 345
 Asp Phe Gly Ser Ala Ala Val Ser Leu Gln Val Ala Ala Pro Tyr Ser
 350 355 360
 35 Lys Pro Ser Met Thr Leu Glu Pro Asn Lys Asp Leu Arg Pro Gly Asp

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The invention also provides isolated nucleic acid compounds that comprise nucleic acid sequences which encode the amino acid compounds provided. Particularly, the isolated nucleic acid compounds that are provided are preferably DNA, however, nucleic acid compounds which are sense or

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antisense mRNA are also provided. A particularly preferred nucleic acid compound is the DNA compound comprising the sequence

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5      agctgtcagc cgcctcacag gaag atg ctg cgt cgg cgg ggc agc cct ggc      51
      Met Leu Arg Arg Arg Gly Ser Pro Gly
      1                      5

      atg ggt gtg cat gtg ggt gca gcc ctg gga gca ctg tgg ttc tgc ctc      99
      Met Gly Val His Val Gly Ala Ala Leu Gly Ala Leu Trp Phe Cys Leu
10      10                      15                      20                      25

      aca gga gcc ctg gag gtc cag gtc cct gaa gac cca gtg gtg gca ctg      147
      Thr Gly Ala Leu Glu Val Gln Val Pro Glu Asp Pro Val Val Ala Leu
      30                      35                      40
15

      gtg ggc acc gat gcc acc ctg tgc tgc tcc ttc tcc cct gag cct ggc      195
      Val Gly Thr Asp Ala Thr Leu Cys Cys Ser Phe Ser Pro Glu Pro Gly
      45                      50                      55

20      ttc agc ctg gca cag ctc aac ctc atc tgg cag ctg aca gat acc aaa      243
      Phe Ser Leu Ala Gln Leu Asn Leu Ile Trp Gln Leu Thr Asp Thr Lys
      60                      65                      70

      cag ctg gtg cac agc ttt gct gag ggc cag gac cag ggc agc gcc tat      291
25      Gln Leu Val His Ser Phe Ala Glu Gly Gln Asp Gln Gly Ser Ala Tyr
      75                      80                      85

      gcc aac cgc acg gcc ctc ttc ccg gac ctg ctg gca cag ggc aac gca      339
      Ala Asn Arg Thr Ala Leu Phe Pro Asp Leu Leu Ala Gln Gly Asn Ala
30      90                      95                      100                      105

      tcc ctg agg ctg cag cgc gtg cgt gtg gcg gac gag ggc agc ttc acc      387
      Ser Leu Arg Leu Gln Arg Val Arg Val Ala Asp Glu Gly Ser Phe Thr
      110                      115                      120
35

      tgc ttc gtg agc atc cgg gat ttc ggc agc gct gcc gtc agc ctg cag      435

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	Cys Phe Val Ser Ile Arg Asp Phe Gly Ser Ala Ala Val Ser Leu Gln	
	125 130 135	
5	gtg gcc gct ccc tac tcg aag ccc agc atg acc ctg gag ccc aac aag Val Ala Ala Pro Tyr Ser Lys Pro Ser Met Thr Leu Glu Pro Asn Lys	483
	140 145 150	
10	gac ctg cgg cca ggg gac acg gtg acc atc acg tgc tcc agc tac cag Asp Leu Arg Pro Gly Asp Thr Val Thr Ile Thr Cys Ser Ser Tyr Gln	531
	155 160 165	
15	ggc tac cct gag gct gag gtg ttc tgg cag gat ggg cag ggt gtg ccc Gly Tyr Pro Glu Ala Glu Val Phe Trp Gln Asp Gly Gln Gly Val Pro	579
	170 175 180 185	
20	ctg act ggc aac gtg acc acg tcg cag atg gcc aac gag cag ggc ttg Leu Thr Gly Asn Val Thr Thr Ser Gln Met Ala Asn Glu Gln Gly Leu	627
	190 195 200	
25	ttt gat gtg cac agc atc ctg cgg gtg gtg ctg ggt gca aat ggc acc Phe Asp Val His Ser Ile Leu Arg Val Val Leu Gly Ala Asn Gly Thr	675
	205 210 215	
30	tac agc tgc ctg gtg cgc aac ccc gtg ctg cag cag gat gcg cac agc Tyr Ser Cys Leu Val Arg Asn Pro Val Leu Gln Gln Asp Ala His Ser	723
	220 225 230	
35	tct gtc acc atc aca ccc cag aga agc ccc aca gga gcc gtg gag gtc Ser Val Thr Ile Thr Pro Gln Arg Ser Pro Thr Gly Ala Val Glu Val	771
	235 240 245	
40	cag gtc cct gag gac ccg gtg gtg gcc cta gtg ggc acc gat gcc acc Gln Val Pro Glu Asp Pro Val Val Ala Leu Val Gly Thr Asp Ala Thr	819
	250 255 260 265	
45	ctg cgc tgc tcc ttc tcc ccc gag cct ggc ttc agc ctg gca cag ctc	867

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	Leu Arg Cys Ser Phe Ser Pro Glu Pro Gly Phe Ser Leu Ala Gln Leu	
	270 275 280	
5	aac ctc atc tgg cag ctg aca gac acc aaa cag ctg gtg cac agt ttc Asn Leu Ile Trp Gln Leu Thr Asp Thr Lys Gln Leu Val His Ser Phe	915
	285 290 295	
10	acc gaa ggc cgg gac cag ggc agc gcc tat gcc aac cgc acg gcc ctc Thr Glu Gly Arg Asp Gln Gly Ser Ala Tyr Ala Asn Arg Thr Ala Leu	963
	300 305 310	
15	ttc ccg gac ctg ctg gca caa ggc aat gca tcc ctg agg ctg cag cgc Phe Pro Asp Leu Leu Ala Gln Gly Asn Ala Ser Leu Arg Leu Gln Arg	1011
	315 320 325	
	gtg cgt gtg gcg gac gag ggc agc ttc acc tgc ttc gtg agc atc cgg Val Arg Val Ala Asp Glu Gly Ser Phe Thr Cys Phe Val Ser Ile Arg	1059
	330 335 340 345	
20	gat ttc ggc agc gct gcc gtc agc ctg cag gtg gcc gct ccc tac tcg Asp Phe Gly Ser Ala Ala Val Ser Leu Gln Val Ala Ala Pro Tyr Ser	1107
	350 355 360	
25	aag ccc agc atg acc ctg gag ccc aac aag gac ctg cgg cca ggg gac Lys Pro Ser Met Thr Leu Glu Pro Asn Lys Asp Leu Arg Pro Gly Asp	1155
	365 370 375	
30	acg gtg acc atc acg tgc tcc agc tac cgg ggc tac cct gag gct gag Thr Val Thr Ile Thr Cys Ser Ser Tyr Arg Gly Tyr Pro Glu Ala Glu	1203
	380 385 390	
35	gtg ttc tgg cag gat ggg cag ggt gtg ccc ctg act ggc aac gtg acc Val Phe Trp Gln Asp Gly Gln Gly Val Pro Leu Thr Gly Asn Val Thr	1251
	395 400 405	
	acg tcg cag atg gcc aac gag cag ggc ttg ttt nnn nnn nnn nnn nnn	1299

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Thr Ser Gln Met Ala Asn Glu Gln Gly Leu Phe Xaa Xaa Xaa Xaa Xaa
 410 415 420 425
 nnn nnn nnn nnn nnn nnn nnn nnn nnn nnn nnn nnn nnn nnn nnn 1347
 5 Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 430 435 440
 nnn nnn nnn nnn nnn nnn gat gcg cac ggc tct gtc acc atc aca ggg 1395
 10 Xaa Xaa Xaa Xaa Xaa Xaa Asp Ala His Gly Ser Val Thr Ile Thr Gly
 445 450 455
 cag cct atg aca ttc ccc cca gag gcc ctg tgg gtg acc gtg ggg ctg 1443
 Gln Pro Met Thr Phe Pro Pro Glu Ala Leu Trp Val Thr Val Gly Leu
 460 465 470
 15 tct gtc tgt ctc att gca ctg ctg gtg gcc ctg gct ttc gtg tgc tgg 1491
 Ser Val Cys Leu Ile Ala Leu Leu Val Ala Leu Ala Phe Val Cys Trp
 475 480 485
 20 aga aag atc aaa cag agc tgt gag gag gag aat gca gga gct gag gac 1539
 Arg Lys Ile Lys Gln Ser Cys Glu Glu Glu Asn Ala Gly Ala Glu Asp
 490 495 500 505
 cag gat ggg gag gga gaa ggc tcc aag aca gcc ctg cag cct ctg aaa 1587
 25 Gln Asp Gly Glu Gly Glu Gly Ser Lys Thr Ala Leu Gln Pro Leu Lys
 510 515 520
 cac tct gac agc aaa gaa gat gat gga caa gaa ata gcc tga 1629
 His Ser Asp Ser Lys Glu Asp Asp Gly Gln Glu Ile Ala
 30 525 530 535
 ccatgaggac cagggagctg ctaccctctcc ctacagctcc taccctctgg ctgc 1683

hereinafter referred to as SEQ ID NO:1.

35 However, an even more preferred isolated nucleic acid compound
 is the DNA compound having the sequence

- 14 -

agctgtcagc cgcctcacag gaag atg ctg cgt cgg cgg ggc agc cct ggc 51
 Met Leu Arg Arg Arg Gly Ser Pro Gly
 1 5
 5
 atg ggt gtg cat gtg ggt gca gcc ctg gga gca ctg tgg ttc tgc ctc 99
 Met Gly Val His Val Gly Ala Ala Leu Gly Ala Leu Trp Phe Cys Leu
 10 15 20 25
 10 aca gga gcc ctg gag gtc cag gtc cct gaa gac cca gtg gtg gca ctg 147
 Thr Gly Ala Leu Glu Val Gln Val Pro Glu Asp Pro Val Val Ala Leu
 30 35 40
 gtg ggc acc gat gcc acc ctg tgc tgc tcc ttc tcc cct gag cct ggc 195
 15 Val Gly Thr Asp Ala Thr Leu Cys Cys Ser Phe Ser Pro Glu Pro Gly
 45 50 55
 ttc agc ctg gca cag ctc aac ctc atc tgg cag ctg aca gat acc aaa 243
 Phe Ser Leu Ala Gln Leu Asn Leu Ile Trp Gln Leu Thr Asp Thr Lys
 20 60 65 70
 cag ctg gtg cac agc ttt gct gag ggc cag gac cag ggc agc gcc tat 291
 Gln Leu Val His Ser Phe Ala Glu Gly Gln Asp Gln Gly Ser Ala Tyr
 75 80 85
 25
 gcc aac cgc acg gcc ctc ttc ccg gac ctg ctg gca cag ggc aac gca 339
 Ala Asn Arg Thr Ala Leu Phe Pro Asp Leu Leu Ala Gln Gly Asn Ala
 90 95 100
 105
 30 tcc ctg agg ctg cag cgc gtg cgt gtg gcg gac gag ggc agc ttc acc 387
 Ser Leu Arg Leu Gln Arg Val Arg Val Ala Asp Glu Gly Ser Phe Thr
 110 115 120
 tgc ttc gtg agc atc cgg gat ttc ggc agc gct gcc gtc agc ctg cag 435
 35 Cys Phe Val Ser Ile Arg Asp Phe Gly Ser Ala Ala Val Ser Leu Gln
 125 130 135

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gtg gcc gct ccc tac tcg aag ccc agc atg acc ctg gag ccc aac aag 483
 Val Ala Ala Pro Tyr Ser Lys Pro Ser Met Thr Leu Glu Pro Asn Lys
 140 145 150

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gac ctg cgg cca ggg gac acg gtg acc atc acg tgc tcc agc tac cag 531
 Asp Leu Arg Pro Gly Asp Thr Val Thr Ile Thr Cys Ser Ser Tyr Gln
 155 160 165

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ggc tac cct gag gct gag gtg ttc tgg cag gat ggg cag ggt gtg ccc 579
 Gly Tyr Pro Glu Ala Glu Val Phe Trp Gln Asp Gly Gln Gly Val Pro
 170 175 180 185

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ctg act ggc aac gtg acc acg tcg cag atg gcc aac gag cag ggc ttg 627
 Leu Thr Gly Asn Val Thr Thr Ser Gln Met Ala Asn Glu Gln Gly Leu
 190 195 200

20

ttt gat gtg cac agc atc ctg cgg gtg gtg ctg ggt gca aat ggc acc 675
 Phe Asp Val His Ser Ile Leu Arg Val Val Leu Gly Ala Asn Gly Thr
 205 210 215

25

tac agc tgc ctg gtg cgc aac ccc gtg ctg cag cag gat gcg cac agc 723
 Tyr Ser Cys Leu Val Arg Asn Pro Val Leu Gln Gln Asp Ala His Ser
 220 225 230

30

tct gtc acc atc aca ccc cag aga agc ccc aca gga gcc gtg gag gtc 771
 Ser Val Thr Ile Thr Pro Gln Arg Ser Pro Thr Gly Ala Val Glu Val
 235 240 245

35

cag gtc cct gag gac ccg gtg gtg gcc cta gtg ggc acc gat gcc acc 819
 Gln Val Pro Glu Asp Pro Val Val Ala Leu Val Gly Thr Asp Ala Thr
 250 255 260 265

ctg cgc tgc tcc ttc tcc ccc gag cct ggc ttc agc ctg gca cag ctc 867
 Leu Arg Cys Ser Phe Ser Pro Glu Pro Gly Phe Ser Leu Ala Gln Leu
 270 275 280

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	aac ctc atc tgg cag ctg aca gac acc aaa cag ctg gtg cac agt ttc	915
	Asn Leu Ile Trp Gln Leu Thr Asp Thr Lys Gln Leu Val His Ser Phe	
	285 290 295	
5		
	acc gaa ggc cgg gac cag ggc agc gcc tat gcc aac cgc acg gcc ctc	963
	Thr Glu Gly Arg Asp Gln Gly Ser Ala Tyr Ala Asn Arg Thr Ala Leu	
	300 305 310	
10		
	ttc ccg gac ctg ctg gca caa ggc aat gca tcc ctg agg ctg cag cgc	1011
	Phe Pro Asp Leu Leu Ala Gln Gly Asn Ala Ser Leu Arg Leu Gln Arg	
	315 320 325	
	gtg cgt gtg gcg gac gag ggc agc ttc acc tgc ttc gtg agc atc cgg	1059
15	Val Arg Val Ala Asp Glu Gly Ser Phe Thr Cys Phe Val Ser Ile Arg	
	330 335 340 345	
	gat ttc ggc agc gct gcc gtc agc ctg cag gtg gcc gct ccc tac tcg	1107
	Asp Phe Gly Ser Ala Ala Val Ser Leu Gln Val Ala Ala Pro Tyr Ser	
20	350 355 360	
	aag ccc agc atg acc ctg gag ccc aac aag gac ctg cgg cca ggg gac	1155
	Lys Pro Ser Met Thr Leu Glu Pro Asn Lys Asp Leu Arg Pro Gly Asp	
	365 370 375	
25		
	acg gtg acc atc acg tgc tcc agc tac cgg ggc tac cct gag gct gag	1203
	Thr Val Thr Ile Thr Cys Ser Ser Tyr Arg Gly Tyr Pro Glu Ala Glu	
	380 385 390	
30		
	gtg ttc tgg cag gat ggg cag ggt gtg ccc ctg act ggc aac gtg acc	1251
	Val Phe Trp Gln Asp Gly Gln Gly Val Pro Leu Thr Gly Asn Val Thr	
	395 400 405	
	acg tcg cag atg gcc aac gag cag ggc ttg ttt gat gtg cac agc gtc	1299
35	Thr Ser Gln Met Ala Asn Glu Gln Gly Leu Phe Asp Val His Ser Val	
	410 415 420 425	

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ctg cgg gtg gtg ctg ggt gcg aat ggc acc tac agc tgc ctg gtg cgc 1347
 Leu Arg Val Val Leu Gly Ala Asn Gly Thr Tyr Ser Cys Leu Val Arg
 430 435 440

5

aac ccc gtg ctg cag cag gat gcg cac ggc tct gtc acc atc aca ggg 1395
 Asn Pro Val Leu Gln Gln Asp Ala His Gly Ser Val Thr Ile Thr Gly
 445 450 455

10

cag cct atg aca ttc ccc cca gag gcc ctg tgg gtg acc gtg ggg ctg 1443
 Gln Pro Met Thr Phe Pro Pro Glu Ala Leu Trp Val Thr Val Gly Leu
 460 465 470

15

tct gtc tgt ctc att gca ctg ctg gtg gcc ctg gct ttc gtg tgc tgg 1491
 Ser Val Cys Leu Ile Ala Leu Leu Val Ala Leu Ala Phe Val Cys Trp
 475 480 485

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aga aag atc aaa cag agc tgt gag gag gag aat gca gga gct gag gac 1539
 Arg Lys Ile Lys Gln Ser Cys Glu Glu Glu Asn Ala Gly Ala Glu Asp
 490 495 500 505

25

cag gat ggg gag gga gaa ggc tcc aag aca gcc ctg cag cct ctg aaa 1587
 Gln Asp Gly Glu Gly Glu Gly Ser Lys Thr Ala Leu Gln Pro Leu Lys
 510 515 520

30

cac tct gac agc aaa gaa gat gat gga caa gaa ata gcc tga 1629
 His Ser Asp Ser Lys Glu Asp Asp Gly Gln Glu Ile Ala
 525 530 535

ccatgaggac cagggagctg ctaccctctcc ctacagctcc taccctctgg ctgc 1683

hereinafter referred to as SEQ ID NO:3.

Also provided by the present invention are nucleic acid vectors
 comprising nucleic acids which encode SEQ ID NO:2 or SEQ ID NO:4 or
 35 functional equivalents thereof. The preferred nucleic acid vectors are those
 which are DNA. Most preferred are DNA vectors which comprise the DNA

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sequence which is SEQ ID NO:3. Moreover, DNA vectors of the present invention preferably comprise a promoter positioned to drive expression of said DNA sequence. Those vectors wherein said promoter functions in human embryonic kidney cells (293 cells), AV12 cells, yeast cells or *E.coli* cells are preferred.

The present invention also provides probes and primers useful for molecular biology techniques. A compound which encodes all or part of SEQ ID NO:2 or SEQ ID NO:4 and which is at least 18 consecutive base pairs in length is provided. Preferably, the 18 base pair or more compound is DNA. Most preferred for this use are the DNA compounds which comprise at least 18 consecutive base pairs of SEQ ID NO:1 or SEQ ID NO:3.

Host cells which harbor the nucleic acids provided by the present invention are also provided. A preferred host cell is an oocyte. A preferred oocyte is one which has been injected with sense mRNA or DNA compounds of the present invention. A more preferred oocyte is one which has been injected with sense mRNA or DNA compounds of the present invention in conjunction with DNA or sense mRNA which encodes APP.

Further, this invention provides cells into which the nucleic acid compounds of the present invention may be transfected. Host cells include those which are transfected with a nucleic acid compound which encodes SEQ ID NO:2 or SEQ ID NO:4. Preferred cells include host cells transfected with a DNA vector comprising SEQ ID NO:1 or SEQ ID NO:3. The preferred transfected host cells which encode SEQ ID NO:2 or SEQ ID NO:4 are 293 cells, AV12 cells, yeast cells and *E. coli* cells. Also preferred is a host cell which has been co-transfected with a DNA vector which comprises SEQ ID NO:1 or SEQ ID NO:3 and a DNA vector which comprises the coding sequence of APP. 293 cells, AV12 cells, yeast cells and *E. coli* cells are the preferred co-transfected host cells.

Additionally, the invention provides a method for identifying DNA homologous to a probe of the present invention, which comprises combining test

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nucleic acid with the probe under hybridizing conditions and identifying those test nucleic acids which hybridize.

Assays utilizing the compounds provided by the present invention are also provided. The assays provided determine whether a substance is a
5 ligand for β -secretase, said method comprising contacting β -secretase with said substance, monitoring β -secretase activity by physically detectable means, and identifying those substances which interact with or affect β -secretase. Preferred assays of the present invention include a cell culture assay, a high-performance liquid chromatography (HPLC) assay or a synthetic competition assay. Pre-
10 ferred cell culture assays utilize oocytes, AV12, *E. coli*, yeast or 293 cells which co-express nucleic acids which encode β -secretase and APP.

The invention also provides methods for constructing a host cell capable of expressing a nucleic acid compound which encodes an amino acid compound comprising SEQ ID NO:2 or SEQ ID NO:4, said methods comprising
15 transfecting a host cell with a DNA vector comprising a nucleic acid compound encoding SEQ ID NO:2 or SEQ ID NO:4. A preferred method utilizes 293, AV12, yeast or *E. coli* cells as the host cells. A more preferred method includes a DNA vector which comprises SEQ ID NO:1 or SEQ ID NO:3. A most preferred method includes a DNA vector which comprises SEQ ID NO:3. Another preferred
20 method comprises (a) a DNA vector which comprises SEQ ID NO:3 and (b) a DNA expression vector which encodes the APP coding sequence.

Additionally, methods for expressing a nucleic acid sequence which encodes SEQ ID NO:2 or SEQ ID NO:4, or functional equivalents thereof, in a transfected host cell, are also provided. These methods comprise culturing a
25 transfected host cell of the present invention under conditions suitable for gene expression. A preferred method utilizes 293, AV12, yeast or *E. coli* cells as the transfected host cell. A more preferred method utilizes a DNA vector to transfect the host cell. A most preferred method utilizes a DNA vector comprising all or part of SEQ ID NO:3.

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The present invention also provides processes for isolating and purifying an amyloid precursor protein protease, said process comprising: (a) establishing in a suitable medium, a culture of the host cells transformed with a polynucleotide encoding an amyloid precursor protein protease; and (b) isolating said protease from said culture. Compositions comprising a peptide isolated according to such processes are also provided.

In addition, polyclonal and monoclonal antibodies to the peptides of the present invention are also provided.

The terms and abbreviations used in this document have their normal meanings unless otherwise designated. For example "°C" refers to degrees Celsius; "N" refers to normal or normality; "mmol" refers to millimole or millimoles; "g" refers to gram or grams; "ml" means milliliter or milliliters; "M" refers to molar or molarity; "μg" refers to microgram or micrograms; "μl" refers to microliter or microliters; "pU" refers to pico Unit, and the like.

All nucleic acid sequences, unless otherwise designated, are written in the direction from the 5' end to the 3' end, frequently referred to as "5' to 3'".

All amino acid or protein sequences, unless otherwise designated, are written commencing with the amino terminus ("N-terminus") and concluding with the carboxy terminus ("C-terminus").

"Base pair" or "bp" as used herein refers to DNA or RNA. The abbreviations A, C, G, and T correspond to the 5'-monophosphate forms of the deoxyribonucleosides (deoxy)adenosine, (deoxy)cytidine, (deoxy)guanosine, and (deoxy)thymidine, respectively, when they occur in DNA molecules. The abbreviations U, C, G, and A correspond to the 5'-monophosphate forms of the ribonucleosides uridine, cytidine, guanosine, and adenosine, respectively when they occur in RNA molecules. In double stranded DNA, base pair may refer to a partnership of A with T or C with G. In a DNA/RNA, heteroduplex

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base pair may refer to a partnership of A with U or C with G. (See the definition of “complementary”, infra.)

The terms “digestion” or “restriction” of DNA refers to the catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA (“sequence-specific endonucleases”). The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors, and other requirements were used as would be known to one of ordinary skill in the art. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer or can be readily found in the literature.

“Ligation” refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments. Unless otherwise provided, ligation may be accomplished using known buffers and conditions with a DNA ligase, such as T4 DNA ligase.

The term “plasmid” refers to an extrachromosomal (usually) self-replicating genetic element. Plasmids are generally designated by a lower case “p” preceded and/or followed by letters and/or numbers. The starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accordance with published procedures. In addition, equivalent plasmids to those described are known in the art and will be apparent to the ordinarily skilled artisan.

The term “reading frame” means the nucleotide sequence from which translation occurs “read” in triplets by the translational apparatus of transfer RNA (tRNA) and ribosomes and associated factors, each triplet corresponding to a particular amino acid. To insure against improper translation, the triplet codons corresponding to the desired polypeptide must be aligned in multiples of three from the initiation codon, i.e. the correct “reading frame” being maintained.

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“Recombinant DNA cloning vector” as used herein refers to any autonomously replicating agent, including, but not limited to, plasmids and phages, comprising a DNA molecule to which one or more additional DNA segments can or have been added.

5 The term “recombinant DNA expression vector” as used herein refers to any recombinant DNA cloning vector in which a promoter to control transcription of the inserted DNA has been incorporated.

 The term “expression vector system” as used herein refers to a recombinant DNA expression vector in combination with one or more trans-
10 acting factors that specifically influence transcription, stability, or replication of the recombinant DNA expression vector. The trans-acting factor may be expressed from a co-transfected plasmid, virus, or other extrachromosomal element, or may be expressed from a gene integrated within the chromosome.

 “Transcription” as used herein refers to the process whereby
15 information contained in a nucleotide sequence of DNA is transferred to a complementary RNA sequence.

 The term “transfection” as used herein refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily
20 skilled artisan, for example, calcium phosphate co-precipitation, and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

 The term “transformation” as used herein means the introduction of DNA into an organism so that the DNA is replicable, either as
25 an extrachromosomal element or by chromosomal integration. Methods of transforming bacterial and eukaryotic hosts are well known in the art, many of which methods, such as nuclear injection, protoplast fusion or by calcium treatment using calcium chloride are summarized in J. Sambrook, et al., MOLECULAR CLONING: A LABORATORY MANUAL, (1989).

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The term "translation" as used herein refers to the process whereby the genetic information of messenger RNA is used to specify and direct the synthesis of a polypeptide chain.

The term "vector" as used herein refers to a nucleic acid compound used for the transformation of cells in gene manipulation bearing polynucleotide sequences corresponding to appropriate protein molecules which when combined with appropriate control sequences confer specific properties on the host cell to be transformed. Plasmids, viruses, and bacteriophage are suitable vectors. Artificial vectors are constructed by cutting and joining DNA molecules from different sources using restriction enzymes and ligases. The term "vector" as used herein includes Recombinant DNA cloning vectors and Recombinant DNA expression vectors.

The terms "complementary" or "complementarity" as used herein refer to the capacity of purine and pyrimidine nucleotides to associate through hydrogen bonding to form double stranded nucleic acid molecules. The following base pairs are related by complementarity: guanine and cytosine; adenine and thymine; and adenine and uracil. As used herein, "complementary" means that the aforementioned relationship applies to substantially all base pairs comprising two single-stranded nucleic acid molecules over the entire length of said molecules. "Partially complementary" refers to the aforementioned relationship in which one of two single-stranded nucleic acid molecules is shorter in length than the other such that a portion of one of the molecules remains single-stranded.

The term "hybridization" as used herein refers to a process in which a strand of nucleic acid joins with a complementary strand through base pairing. The conditions employed in the hybridization of two completely or nearly completely complementary nucleic acid strands varies with the degree of complementarity of the two strands and the length of the strands. Such techniques and conditions are well known to practitioners in this field.

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“Isolated amino acid sequence” refers to any amino acid sequence, however constructed or synthesized, which is locationally distinct from the naturally occurring sequence.

5 “Isolated DNA compound” refers to any DNA sequence, however constructed or synthesized, which is locationally distinct from its natural location in genomic DNA.

“Isolated nucleic acid compound” refers to any RNA or DNA sequence, however constructed or synthesized, which is locationally distinct from its natural location.

10 A “primer” is a nucleic acid fragment which functions as an initiating substrate for enzymatic or synthetic elongation.

The term “promoter” refers to a DNA sequence which directs transcription of DNA to RNA.

15 A “probe” as used herein is a nucleic acid compound or a fragment thereof which hybridizes with a nucleic acid compound which encodes either the entire sequence of SEQ ID NO:2 or SEQ ID NO:4, a sequence complementary to SEQ ID NO:2 or SEQ ID NO:4, or a part thereof.

The term “EST” or “expressed sequence tag” refers to a fragment or sampling of cDNA which encodes a polypeptide of unknown function.

20 The term “stringency” refers to a set of hybridization conditions which may be varied in order to vary the degree of nucleic acid affinity for other nucleic acid. (See the definition of “hybridization”, supra.)

25 The term “antigenically distinct” as used herein refers to a situation in which antibodies raised against an epitope of the proteins of the present invention, or a fragment thereof, may be used to differentiate between the proteins of the present invention and other β -secretase variants. This term may also be employed in the sense that such antibodies may be used to differentiate between the human β -secretase protein and analogous proteins derived from other species.

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The term "PCR" as used herein refers to the widely-known polymerase chain reaction employing a thermally-stable polymerase. This technique, as appreciated by those skilled in the art, is employed to amplify a particular nucleic acid fragment.

5 The term "BLAST" as used herein refers to the widely known basic local alignment search tool. This tool consists of a set of computer-based programs designed to permit examination of amino acid and nucleic acid sequence databases for similarity with an isolated sequence of interest.

10 The term "RACE" refers to the widely known rapid amplification of complimentary ends technique, to amplify and obtain the 5' and 3' ends of isolated cDNA.

 Skilled artisans will recognize that the proteins of the present invention can be synthesized by a number of different methods. All of the amino acid compounds of the invention can be made by chemical methods well known in the art, including solid phase peptide synthesis, or
15 recombinant methods. Both methods are described in U.S. Patent 4,617,149, herein incorporated by reference.

 The principles of solid phase chemical synthesis of polypeptides are well known in the art and may be found in general texts in the area. See,
20 e.g., H. Dugas and C. Penney, BIOORGANIC CHEMISTRY, (1981) Springer-Verlag, New York, pgs. 54-92. For examples, peptides may be synthesized by solid-phase methodology utilizing an Applied Biosystems 430A peptide synthesizer (commercially available from Applied Biosystems, Foster City California) and synthesis cycles supplied by Applied Biosystems. Protected
25 amino acids, such as *t*-butoxycarbonyl-protected amino acids, and other reagents are commercially available from many chemical supply houses.

 Sequential *t*-butoxycarbonyl chemistry using double couple protocols are applied to the starting *p*-methyl benzhydryl amine resins for the production of C-terminal carboxamides. For the production of C-terminal
30 acids, the corresponding pyridine-2-aldoxime methiodide resin is used.

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Asparagine, glutamine, and arginine are coupled using preformed hydroxy benzotriazole esters. The following side chain protection may be used:

- Arg, Tosyl
- Asp, cyclohexyl
- 5 Glu, cyclohexyl
- Ser, Benzyl
- Thr, Benzyl
- Tyr, 4-bromo carbobenzoxy

Removal of the *t*-butoxycarbonyl moiety (deprotection) may be
10 accomplished with trifluoroacetic acid (TFA) in methylene chloride.
Following completion of the synthesis the peptides may be deprotected and
cleaved from the resin with anhydrous hydrogen fluoride containing 10%
meta-cresol. Cleavage of the side chain protecting group(s) and of the peptide
from the resin is carried out at zero degrees centigrade or below, preferably -
15 20°C for thirty minutes followed by thirty minutes at 0°C.

After removal of the hydrogen fluoride, the peptide/resin is
washed with ether, and the peptide extracted with glacial acetic acid and
then lyophilized. Purification is accomplished by size-exclusion
chromatography on a Sephadex G-10 (Pharmacia) column in 10% acetic acid.

20 The proteins of the present invention may also be produced by
recombinant methods. Recombinant methods are preferred if a high yield is
desired. A general method for the construction of any desired DNA sequence
is provided in J. Brown, et al., Methods in Enzymology, 68:109 (1979). See
also, J. Sambrook, et al., supra.

25 The basic steps in the recombinant production of desired
proteins are:

- a) construction of a synthetic or semi-synthetic DNA
encoding the protein of interest;

30

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b) integrating said DNA into an expression vector in a manner suitable for the expression of the protein of interest, either alone or as a fusion protein;

5 c) transforming an appropriate eukaryotic or prokaryotic host cell with said expression vector,

d) culturing said transformed or transfected host cell in a manner to express the protein of interest; and

10

e) recovering and purifying the recombinantly produced protein of interest.

In general, prokaryotes are used for cloning of DNA sequences in constructing the vectors of this invention. Prokaryotes may also be employed in the production of the protein of interest. For example, the Escherichia coli K12 strain 294 (ATCC No. 31446) is particularly useful for the prokaryotic expression of foreign proteins. Other strains of E. coli which may be used (and their relevant genotypes) include the following.

20

Strain	Genotype
DH5a	F ⁻ (j80dlacZDM15), D(lacZYA-argF)U169 supE44, l ⁻ , hsdR17(r _K ⁻ , m _K ⁺), recA1, endA1, gyrA96, thi-1, relA1
HB101	supE44, hsdS20(r _B ⁻ m _B ⁻), recA13, ara-14, proA ₂ lacY1, galK2, rpsL20, xyl-5, mtl-1, mcrB, mrr

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JM109	recA1, e14 ⁻ (mcrA), supE44, endA1, hsdR17(r _K ⁻ , m _K ⁺), gyrA96, relA1, thi-1, (lac- proAB), F'[traD36, proAB+ lacI ^q , lacZ [?] M15]
RR1	supE44, hsdS20(r _B ⁻ m _B ⁻), ara-14 proA ₂ , lacY1, galK2, rpsL20, xyl-5, mtl-5
c1776	F ⁻ , ton, A53, dapD8, minA1, supE42 (glnV42), D(gal-uvrB)40, minB2, rfb-2, gyrA ₂₅ , thyA142, oms-2, metC65, oms-1, D(bioH-asd)29, cycB2, cycA1, hsdR2, l ⁻
294	endA, thi ⁻ , hsr ⁻ , hsm _K ⁺ (U.S. Patent 4,366,246)
LE392	F ⁻ , hsdR514 (r ⁻ m ⁻), supE44, supF58, lacY1, or Dlac(I-Y)6, galK2, glaT22, metB1, trpR55, l ⁻

These strains are all commercially available from suppliers such as: Bethesda Research Laboratories, Gaithersburg, Maryland 20877 and Stratagene Cloning Systems, La Jolla, California 92037; or are readily

5 available to the public from sources such as the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, 10852-1776. Except where otherwise noted, these bacterial strains can be used interchangeably. The genotypes listed are illustrative of many of the desired characteristics for choosing a bacterial host and are not meant to limit the invention in any way.

10 The genotype designations are in accordance with standard nomenclature. See, for example, J. Sambrook, *et al.*, supra.

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In addition to the strains of E. coli discussed supra, bacilli such as Bacillus subtilis, other enterobacteriaceae such as Salmonella typhimurium or Serratia marcescans, and various Pseudomonas species may be used. In addition to these gram-negative bacteria, other bacteria, especially Streptomyces, spp., may be employed in the prokaryotic cloning and expression of the proteins of this invention.

Promoters suitable for use with prokaryotic hosts include the b-lactamase [vector pGX2907 (ATCC 39344) contains the replicon and b-lactamase gene] and lactose promoter systems [Chang et al., Nature (London), 275:615 (1978); and Goeddel et al., Nature (London), 281:544 (1979)], alkaline phosphatase, the tryptophan (trp) promoter system [vector pATH1 (ATCC 37695) is designed to facilitate expression of an open reading frame as a trpE fusion protein under control of the trp promoter] and hybrid promoters such as the tac promoter (isolatable from plasmid pDR540 ATCC-37282). However, other functional bacterial promoters, whose nucleotide sequences are generally known, enable one of skill in the art to ligate them to DNA encoding the proteins of the instant invention using linkers or adapters to supply any required restriction sites. Promoters for use in bacterial systems also will contain a Shine-Dalgarno sequence operably linked to the DNA encoding the desired polypeptides. These examples are illustrative rather than limiting.

The proteins of this invention may be synthesized either by direct expression or as a fusion protein comprising the protein of interest as a translational fusion with another protein or peptide which may be removable by enzymatic or chemical cleavage. It is often observed in the production of certain peptides in recombinant systems that expression as a fusion protein prolongs the lifespan, increases the yield of the desired peptide, or provides a convenient means of purifying the protein of interest. A variety of peptidases (e.g. trypsin) which cleave a polypeptide at specific sites or digest the peptides from the amino or carboxy termini (e.g. diaminopeptidase) of the peptide

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chain are known. Furthermore, particular chemicals (e.g. cyanogen bromide) will cleave a polypeptide chain at specific sites. The skilled artisan will appreciate the modifications necessary to the amino acid sequence (and synthetic or semi-synthetic coding sequence if recombinant means are employed) to incorporate site-specific internal cleavage sites. See e.g., P. Carter, "Site Specific Proteolysis of Fusion Proteins", Chapter 13 in PROTEIN PURIFICATION: FROM MOLECULAR MECHANISMS TO LARGE SCALE PROCESSES, American Chemical Society, Washington, DC (1990).

In addition to cloning and expressing the genes of interest in the prokaryotic systems discussed above, the proteins of the present invention may also be produced in eukaryotic systems. The present invention is not limited to use in a particular eukaryotic host cell. A variety of eukaryotic host cells are available from depositories such as the American Type Culture Collection (ATCC) and are suitable for use with the vectors of the present invention. The choice of a particular host cell depends to some extent on the particular expression vector used to drive expression of the amyloid precursor protein protease-encoding nucleic acids of the present invention. Exemplary host cells suitable for use in the present invention are listed in Table I. Theses exemplary host cells are merely illustrative of the many eukaryotic cells available for use with the present invention and are not meant in any way to limit the scope of the present invention.

Table I

Host Cell	Origin	Source
HepG-2	Human Liver Hepatoblastoma	ATCC HB 8065
CV-1	African Green Monkey Kidney	ATCC CCL 70
LLC-MK ₂	Rhesus Monkey Kidney	ATCC CCL 7
3T3	Mouse Embryo Fibroblasts	ATCC CCL 92
CHO-K1	Chinese Hamster Ovary	ATCC CCL 61
HeLa	Human Cervix Epitheloid	ATCC CCL 2

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RPMI8226	Human Myeloma	ATCC CCL 155
H4IIEC3	Rat Hepatoma	ATCC CCL 1600
C127I	Mouse Fibroblast	ATCC CCL 1616
293	Human Embryonal Kidney	ATCC CRL 1573
Sf9	Fall armyworm ovary <u>Spodoptera</u>	ATCC CRL-1711
	<u>frugiperda</u>	
HS-Sultan	Human Plasma Cell Plasmocytoma	ATCC CCL 1484
BHK-21	Baby Hamster Kidney	ATCC CCL 10

A preferred cell line employed in the expression of the protein of the present invention is the widely available 293 cell line. As noted, this cell
 5 line was constructed from human embryonal kidney tissue and is available from American Type Culture Collection under the accession number ATCC CCL 1573.

A wide variety of vectors, some of which are discussed below, exists for the transformation of mammalian host cells, but the specific vectors
 10 described herein are merely illustrative and are in no way intended to limit the scope of the present invention.

Some illustrative vectors include the pSV2-type vectors which comprise segments of the simian virus 40 (SV40) genome that constitute a defined eukaryotic transcription unit-promoter, intervening sequence, and
 15 polyadenylation site. In the absence of the SV40 T antigen, the plasmid pSV2-type vectors transform mammalian and other eukaryotic host cells by integrating into the host cell chromosomal DNA. A large number of plasmid pSV2-type vectors have been constructed, such as plasmid pSV2-gpt, pSV2-neo, pSV2-dhfr, pSV2-hyg, and pSV2-b-globin, in which the SV40 promoter
 20 drives transcription of an inserted gene. These vectors are suitable for use with the coding sequences of the present invention and are widely available

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from sources such as the ATCC or the Northern Regional Research Laboratory (NRRL), 1815 N. University Street, Peoria, Illinois, 61604.

The plasmid pSV2-dhfr (ATCC 37146) comprises a murine dihydrofolate reductase (dhfr) gene under the control of the SV40 early promoter. Under the appropriate conditions, the dhfr gene is known to be amplified, or copied, in the host chromosome. This amplification can result in the amplification of closely-associated DNA sequences and can, therefore, be used to increase production of a protein of interest. See, e.g., J. Schimke, Cell, 35:705-713 (1984).

Plasmids constructed for expression of the proteins of the present invention in mammalian and other eukaryotic host cells can utilize a wide variety of promoters. The present invention is in no way limited to the use of the particular promoters exemplified herein. Promoters such as the SV40 late promoter, promoters from eukaryotic genes, such as, for example, the estrogen-inducible chicken ovalbumin gene, the interferon genes, the gluco-corticoid-inducible tyrosine aminotransferase gene, and the thymidine kinase gene, and the major early and late adenovirus genes can be readily isolated and modified to express the genes of the present invention. Eukaryotic promoters can also be used in tandem to drive expression of a coding sequence of this invention. Furthermore, a large number of retroviruses are known that infect a wide range of eukaryotic host cells. The long terminal repeats in the retroviral DNA frequently encode functional promoters and, therefore, may be used to drive expression of the nucleic acids of the present invention.

Plasmid pRSVcat (ATCC 37152) comprises portions of a long terminal repeat of the Rous Sarcoma virus, a virus known to infect chickens and other host cells. This long terminal repeat contains a promoter which is suitable for use in the vectors of this invention. H. Gorman, et al., Proceedings of the National Academy of Sciences (USA), 79:6777 (1982). The plasmid pMSVi (NRRL B-15929) comprises the long terminal repeats of the

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Murine Sarcoma virus, a virus known to infect mouse and other host cells.

The mouse metallothionein promoter has also been well characterized for use in eukaryotic host cells and is suitable for use in the expression of the nucleic acids of the present invention. The mouse metallothionein promoter is

5 present in the plasmid pdBPV-MMTneo (ATCC 37224) which can serve as the starting material of other plasmids of the present invention.

Another usefull expression vector system employs one of a series of vectors containing the BK enhancer, an enhancer derived from the BK virus, a human papovavirus. The most preferred such vector systems are
10 those which employ not only the BK enhancer but also the adenovirus-2-early region 1A (E1A) gene product. The E1A gene product (actually, the E1A gene produces two products, which are collectively referred to herein as "the E1A gene product") is an immediate-early gene product of adenovirus, a large DNA virus.

15 An additional useful eukaryotic expression vector is the phd series of vectors which comprise a BK enhancer in tandem with the adenovirus late promoter to drive expression of useful products in eukaryotic host cells. The construction and method of using the phd plasmid, as well as related plasmids, are described in U.S. Patents 5,242,688, issued September
20 7, 1993, and 4,992,373, issued February 12, 1991, all of which are herein incorporated by reference. Escherichia coli K12 GM48 cells harboring the plasmid phd are available as part of the permanent stock collection of the Northern Regional Research Laboratory under accession number NRRL B-18525. The plasmid may be isolated from this culture using standard
25 techniques.

The plasmid phd contains a unique BclI site which may be utilized for the insertion of the gene encoding the protein of interest. The skilled artisan understands that linkers or adapters may be employed in cloning the gene of interest into this BclI site. The phd series of plasmids
30 functions most efficiently when introduced into a host cell which produces the

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E1A gene product, cell lines such as AV12-664, 293 cells, and others, described supra.

Transformation of the mammalian cells can be performed by any of the known processes including, but not limited to, the protoplast fusion method, the calcium phosphate co-precipitation method, electroporation and the like. See, e.g., J. Sambrook, et al., supra, at 3:16.30-3:16.66.

Other routes of production are well known to skilled artisans. In addition to the plasmid discussed above, it is well known in the art that some viruses are also appropriate vectors. For example, the adenovirus, the adeno-associated virus, the vaccinia virus, the herpes virus, the baculovirus, and the rous sarcoma virus are useful. Such a method is described in U.S. Patent 4,775,624, herein incorporated by reference. Several alternate methods of expression are described in J. Sambrook, et al., supra, at 16.3-17.44.

In addition to prokaryotes and mammalian host cells, eukaryotic microbes such as yeast cultures may also be used. The imperfect fungus Saccharomyces cerevisiae, or common baker's yeast, is the most commonly used eukaryotic microorganism, although a number of other strains are commonly available. For expression in Saccharomyces sp., the plasmid YRp7 (ATCC-40053), for example, is commonly used. See, e.g., L. Stinchcomb, et al., Nature (London), 282:39 (1979); J. Kingsman et al., Gene, 7:141 (1979); S. Tschemper et al., Gene, 10:157 (1980). This plasmid already contains the trp gene which provides a selectable marker for a mutant strain of yeast lacking the ability to grow in tryptophan.

Suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase [found on plasmid pAP12BD (ATCC 53231) and described in U.S. Patent No. 4,935,350, issued June 19, 1990, herein incorporated by reference] or other glycolytic enzymes such as enolase [found on plasmid pAC1 (ATCC 39532)], glyceraldehyde-3-phosphate dehydrogenase [derived from plasmid pHcGAPC1 (ATCC 57090, 57091)], hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-

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phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase, as well as the alcohol dehydrogenase and pyruvate decarboxylase genes of Zymomonas mobilis (United States Patent No. 5,000,000 issued March 19, 1991, herein incorporated by reference).

Other yeast promoters, which are inducible promoters, having the additional advantage of their transcription being controllable by varying growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein [contained on plasmid vector pCL28XhoLHBPV (ATCC 39475) and described in United States Patent No. 4,840,896, herein incorporated by reference], glyceraldehyde 3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose [e.g. GAL1 found on plasmid pRY121 (ATCC 37658)] utilization. Suitable vectors and promoters for use in yeast expression are further described in R. Hitzeman et al., European Patent Publication No. 73,657A. Yeast enhancers such as the UAS Gal from Saccharomyces cerevisiae (found in conjunction with the CYC1 promoter on plasmid YEpsec--h11beta ATCC 67024), also are advantageously used with yeast promoters.

Practitioners of this invention realize that, in addition to the above-mentioned expression systems, cloned cDNA may also be employed in the production of transgenic animals in which a test mammal, usually a mouse, in which expression or overexpression of the proteins of the present invention can be assessed. The nucleic acids of the present invention may also be employed in the construction of "knockout" animals in which the expression of the native cognate of the gene is suppressed.

Skilled artisans will recognize that the protease of the present invention, as well as the nucleic acid compounds encoding said protease, can be isolated and purified from cultured host cells transiently expressing β -secretase activity. The following examples further illustrate such isolation

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and purification processes, however, these examples are not in any way to be interpreted as limiting the scope of the present invention.

Isolation and Purification of β -secretase

5

Example 1

293 Cell Source

293 Cells were obtained as by-products from 25- to 30-day fermentations at Lilly Technology Center. (Eli Lilly and Co., Indianapolis, IN.)

10

Example 2

β -Secretase Activity Assay

The enzymatic activity of beta-secretase was determined by an ELISA assay, modified in form over an assay appearing in application No. PCT/US96/09985, the entire content of which is herein incorporated by reference. Using MBP-APPc125sw as the substrate, the activity was determined by measuring the MBP-APPc26sw cleavage product with a polyclonal antibody, 192sw, highly specific to the product. MBP-APPc125sw is a fusion substrate in which maltose binding protein is fused to the 125-residue C-terminal portion of the swedish amyloid precursor protein. For the enzymatic activity analysis, one picounit (pU) is defined as the amount of the enzyme that catalyzes the conversion of MBP-APP125sw to one picomole of MBP-APPc26sw per ml in 2-hr under the optimized reaction conditions of the Elisa assay (see Table II, infra). To calculate the enzyme specific activity, the protein concentration was first determined using the well known Bio-Rad microassay method. (Bio-Rad Laboratories, Hercules, CA, 94547)

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Example 3**β-Secretase Purification**

5 The purification of beta-secretase was carried out by a protocol modified over the purification methods appearing in application No. PCT/US96/09985, the entire content of which is herein incorporated by reference. The enzyme purification protocol was undertaken at 4 C. β-Secretase activity and protein recovery data are provided in Table III, *infra*.

Step 1:

10 **Membrane Extract:** About 400-g of 293 cells were resuspended in 2,000-ml of 20 mM HEPES (Sigma Chemical Co., St Louis, MO), pH 7.5, 2 mM EDTA (Sigma Chemical Co., St Louis, MO) and 0.25% sucrose (Buffer 1A). The cell
15 suspension was homogenized in an 1000-ml aliquot and the cells were broken with an Brinkmann Homogenizer (POLYTRON, Switzerland) according to the following conditions: at setting 4 for 2- and 1-min; and at setting 5 for 1- and 0.5-min (with 3-min cooling time after each homogenization). The broken cell suspension was centrifuged at 1,000xg for 20-min; the resulting
20 supernatant (post-nuclear supernatant; PNS1) was saved. The pellet was resuspended in 1,200-ml of Buffer 1A, homogenized, broken and centrifuged as described above; the supernatant (PNS2) was saved and the pellet was discarded. PNS1 and PNS2 were combined (2,700-ml) and centrifuged at 45,000xg for 1-hr; the resulting pellet (P2; containing membranes) was saved
25 and the supernatant was discarded. This pellet was resuspended in 3,200-ml of 20 mM MES (Sigma Chemical Co., St Louis, MO), pH 6.0, 2 mM EDTA, 0.5% Triton X-100, 150 mM NaCl, 0.2 mM AEBSF, 5 mg/ml leupeptin, 2 mg/ml E64 and 1 mg/ml pepstatin A (Buffer 1B); all four protease inhibitors were from Calbiochem; San Diego, CA). The suspension was stirred
30 continuously for 1-hr for extraction of beta-secretase and centrifuged at

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16,000xg for 1-hr; the pellet was discarded. The resulting supernatant (3,120-ml) was adjusted to pH 7.5 with 1 M Tris base and filtered through 0.45-mm Zapcap-S (Schleicher & Schuell; Keene, NH); the filtrate was the P2 membrane extract (P2ME, 3,170-ml).

5

A second P2ME was prepared similarly from another 400g of 293 cells. The combined beta-secretase activity and protein data for the P2ME fractions are shown in Table III. However, beta-secretase activity from each P2ME was purified separately in the next step.

10

Step 2:

Wheat Germ Agglutinin (WGA) Agarose Eluate: The first P2ME was loaded to a WGA-Agarose column (Vector Lab.; Burlingame, CA) (1000-ml), pre-equilibrated with 1500-ml of 20 mM Tris, pH 7.5, 2 mM EDTA, 0.5% Triton X-100 and 150 mM NaCl (Buffer 2A). The WGA column, which binds to N-acetyl-D-glucosamine glycopeptides, was washed with 1000-ml of Buffer 2A and bound proteins were eluted with a linear gradient of 0-10% chitin hydrolysate (Vector Lab.) in a total volume of 900-ml constructed from Buffer 2A and Buffer 2B (20 mM Tris, pH 7.5, 2 mM EDTA, 0.5% Triton X-100 and 10% chitin hydrolysate). The fractions containing higher specific activities of beta-secretase were pooled as the WGA-eluate (180-ml).

The WGA-Agarose column was regenerated and equilibrated. The second WGA-eluate (170-ml) was obtained similarly from the second P2ME. The two WGA-eluates were combined.(see Table III). In addition, the two WGA-Agarose washings were combined.(see Table III). The WGA-eluate and the WGA-wash were purified together in the next step.

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Step 3:

HiTrap Q (HiQ) Eluate: The WGA-eluate and the WGA-wash were diluted 6x and 4x, respectively, with 20 mM Tris, pH 8.0, 2 mM EDTA and 0.2% Triton X-100 (Buffer 3A) to reduce Triton X-100 and NaCl concentrations. Both diluted enzyme preparations were loaded consecutively to four connected, fast flow anion exchanger HiTrap Q columns (4x5-ml; Amersham Pharmacia Biotech, Piscataway, NJ), pre-equilibrated with 100-ml of Buffer 3A, and the column was washed with 200-ml of Buffer 3A. Bound proteins were eluted first with a linear gradient of 0-500 mM NaCl in buffer 3A in a total volume of 420-ml and then eluted with 100-ml of Buffer 3B (Buffer 3A plus 500 mM NaCl). The fractions containing higher specific activities were pooled as the HiQ-eluate. (Table III) The HiQ-eluate was used directly in the next step.

15

Step 4:

Hydroxylapatite (HA) Wash: The HiQ-eluate was loaded to a Hydroxylapatite column (10-ml; Bio-Rad, Hercules, CA), pre-equilibrated with 100-ml of Buffer 4A (same as Buffer 3A), the column flow-through was collected. HA separation of proteins is based on charge absorption and desorption. The column was washed with 50-ml of Buffer 4A, and the bound proteins were eluted from the column with a linear gradient of 0-500 mM potassium phosphate in Buffer 4A in a total volume of 100-ml. Among the three fractions (the flow-through, the wash, and the eluate), the HA-wash (50-ml) showed the highest total , and specific activity of beta-secretase (Table III) and, thus, was used in the next step.

25

Step 5:

Mono Q Eluate. The HA-wash was de-salted to about 15 mM NaCl with Buffer 5A (same as Buffer 4A) using Centriprep 50 (Millipore, Bedford, MA).

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- 40 -

The de-salted HA-wash was loaded to a high resolving strong anion exchanger Mono Q column (1-ml; Amersham Pharmacia Biotech), pre-equilibrated with 30-ml of Buffer 5A. The column was washed with 30-ml of buffer 5A, and bound proteins were eluted first with a linear gradient of 0-
5 500 mM in Buffer 5A in a total volume of 20-ml and then eluted with 10-ml of Buffer 5B (Buffer 5A plus 500 mM NaCl). Two activity peaks were observed and the active fractions from each activity peak were pooled.(Table III) The two Mono Q-eluates (2-ml & 4-ml)were purified separately in the next step.

10 Step 6:

Superdex (S) 200 Eluate. Mono Q-eluate 1 was loaded to a high resolution gel-filtration Superdex 200 column (300-ml; Amersham Pharmacia Biotech), pre-equilibrated with 900-ml of 20 mM Tris, pH 8.0, 2 mM EDTA, 0.2% Triton X-100, 1 mM MgCl₂, 1 mM CaCl₂ and 150 mM NaCl (Buffer 6). The
15 proteins were eluted with 300-ml of Buffer 6. Two activity peaks were observed and the active fractions from both peaks were pooled together (S-200-eluate 1, 19.2-ml)(Table III) and used in the next step. Mono Q-eluate 2 was similarly purified by the same Superdex 200 column; three activity peaks were observed. The two Superdex 200-eluates that showed the higher specific
20 activities (S-200 eluates 2a & 2b; Table III) were used separately in the next step.

Step 7:

DEAE Eluate. S-200-eluates 1 and 2a were de-salted with a Sepharose G-25
25 column (100-ml; Amersham Pharmacia Biotech) and S-200-eluate 2b was diluted 6x with Buffer 7A (same as 5A) to 25 mM NaCl.. To the resulting S-200-eluates; 200-, 200- and 100 µl of DEAE-Sepharose was added, respectively. For effective binding of the activity to the resin, the three samples were agitated continuously overnight. The samples were centrifuged
30 at 10,000xg for 10-min to collect DEAE-Sepharose beads. The bound proteins

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were eluted from DEAE-Sepharose beads of the three samples with, respectively, 2x200-, 2x400- and 2x100 µl of Buffer 7B (Buffer 7A plus 1 M NaCl). The three DEAE-eluates (400-, 800-, and 200 µl; Table III) were treated separately in the next step.

5

Step 8:

De-Glycosylation. To each of the three DEAE-eluates, beta-mercaptoethanol (reducing agent) was added to a final concentration of 3 mM. PNGase F (100 mU/40-ml from Glyko, Novato, CA); an N-linked de-glycosylation enzyme, was added as three aliquots to each sample to a final ratio of about 1:4 (V/V). After each PNGase addition, the samples were placed in a 37 °C incubator for one day. After a total incubation time of three days, beta-secretase activity was stable in two samples and there was a slight (about 20%) activity loss in the other sample (Table III). The three samples (De-Gly) were purified separately in the next and last step.

15

Step 9:

Mini Q Eluate. Each of the three De-Gly samples was de-salted with a Sephadex PD-10 column (Amersham Pharmacia Biotech). Desalted De-Gly sample 1 was loaded to a Mini Q PE column (0.8-ml from Amersham Pharmacia Biotech), a high resolving anion exchanger, pre-equilibrated with Buffer 9 A (same as Buffer 7A). The bound proteins were washed and eluted with a step-wise NaCl gradient in Buffer 7A in a total volume of 35.6-ml as shown below: 7.6-ml at 0 mM NaCl, 16-ml from 0 to 250 mM, 8-ml from 250 to 500 mM and 4-ml at 500 mM. Two activity peaks were observed. Desalted De-Gly 2a were purified in a similar way; two activity peaks were also observed. Desalted De-Gly 2b sample was purified with a Mini Q PC column (0.24-ml from Amersham Pharmacia Biotech) using a step-wise NaCl gradient in Buffer 7A in a total volume of 13.2-ml (4.8-ml at 0 mM NaCl, 4.8-ml from 0 to 250 mM, 2.4-ml from 250 to 500 mM and 1.2-ml at 500 mM).

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Only one major activity peak was observed. The resulting beta-secretase activity and protein data are shown in Table III.

Beta-secretase activity was purified up to 3,000-fold to apparent homogeneity by the 9-step procedure. Note, however, that the actual activity enrichment was likely much greater excluding Triton X-100 interference to the low level protein determination in Step 9 above.

Table II

Optimized Assay for β -Secretase Activity

Assay Parameter	Optimized Activity Conditions
Enzyme*	0.015-0.095 pU for Linear Response (With 0.02 to 0.06 % Triton X-100)
Substrate	~60 nM for Maximal Activity (With ~20 mM Guanidine HCl)
pH	5.25 to 5.75 for Maximal Activity
Reaction Time	≥ 4 hours for Linear Response
Kinetic Constants	
K_m	300 nM
V_{max}	2 nmol/hr/mg protein

*Using 0.313- μ l of Mini Q-Eluate 2a .

Note: The enzymatic reaction was carried out at room temperature. The purified enzyme was stable at 4 or -20 C for at least two weeks.

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Table III

	Fraction (step)	Total Activity (pU)	Total Protein (mg)	Sp. Act. (pU/mg)	Recovery (%)
5	P2ME (1)	35,038	23,245	1.507	100
	WGA-Wash (2)	2,160	206	10.49	6
10	WGA-E* (2)	8,330	652	12.78	24
	HiQ-E* (3)	5,998	259	23.16	17
	HA-Wash (4)	2,550	20.4	125.0	7.3
15	Mono Q-E*1 (5)	1,248	3.99	312.7	3.6
	Mono Q-E*2 (5)	2,240	6.43	348.4	6.4
	S-200-E*1 (6)	199.5	0.90	221.7	0.6
20	S-200-E*2b (6)	386.5	1.54	251.0	1.1
	S-200-E*2c (6)	60.0	0.16	375.0	0.2
	DEAE-E*1 (7)	239.3	0.406	574.5	0.7
	DEAE-E*2 (7)	771.8	0.642	1202	2.2
25	DEAE-E*3 (7)	8.35	0.048	174.0	---
	De-Gly*1 (8)	222.5	0.397	560.5	0.6
	De-Gly*2 (8)	534.3	0.634	842.7	1.5
	De-Gly*3 (8)	11.33	0.046	246.2	---
30	Mini Q-E*1a (9)	121.8	0.049	2485	0.3
	Mini Q-E*1b (9)	76.8	0.045	1706	0.2
	Mini Q-E*2a (9)	247.0**	0.087	2839	0.7
	Mini Q-E*2b (9)	369.8**	0.121	3056	1.1
35	Mini Q-E*3 (9)	2.95	0.009	327.8	---

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*E = Eluate; FT = Flow-Through; De-Gly = De-Glycosylated

** Mini Q-E 1a and Mini Q-E 1b each contained two activity peaks.

5 Mini Q-Eluate 2a represents the first peak from fraction Q-E 1a combined with the first peak from fraction Q-E 1b.

Mini Q-Eluate 2b represents the second peak from fraction Q-E 1a combined with the second peak from fraction Q-E 1b.

10

Skilled artisans will recognize that some alterations of SEQ ID NO:2 or SEQ ID NO:4 will fail to change the function of the amino acid compound. For instance, some hydrophobic amino acids may be exchanged for other hydrophobic amino acids. Those altered amino acid compounds
15 which confer substantially the same function in substantially the same manner as the exemplified amino acid compounds are also encompassed within the present invention. Typical such conservative substitutions attempt to preserve the: (a) secondary or tertiary structure of the polypeptide backbone; (b) the charge or hydrophobicity of the residue; or (c) the bulk of
20 the side chain. Some examples of such conservative substitutions of amino acids, resulting in the production of proteins which are functional equivalents of the protein of SEQ ID NO:2 or SEQ ID NO:4 are shown in Table II, infra.

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Table IV

	Original Residue	Exemplary Substitutions
	Ala	Ser, Gly
	Arg	Lys
5	Asn	Gln, His
	Asp	Glu
	Cys	Ser
	Gln	Asn
	Glu	Asp
10	Gly	Pro, Ala
	His	Asn, Gln
	Ile	Leu, Val
	Leu	Ile, Val
	Lys	Arg, Gln, Glu
15	Met	Leu, Ile
	Phe	Met, Leu, Gyr
	Ser	Thr
	Thr	Ser
	Trp	Tyr
20	Tyr	Trp, Phe
	Val	Ile, Leu

These substitutions may be introduced into the protein in a variety of ways, such as during the chemical synthesis or by chemical modification of an amino acid side chain after the protein has been prepared.

Alterations of the protein having a sequence which corresponds to the sequence of SEQ ID NO:2 or SEQ ID NO:4 may also be induced by alterations of the nucleic acid compounds which encodes these proteins. These mutations of the nucleic acid compound may be generated by either

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random mutagenesis techniques, such as those techniques employing chemical mutagens, or by site-specific mutagenesis employing oligonucleotides. In addition, allelic variants of the gene encoding the protein of the present invention may also be purified by the processes of the present invention. Those nucleic acid compounds which confer substantially the same function in substantially the same manner as the exemplified nucleic acid compounds are also encompassed within the present invention.

Other embodiments of the present invention include nucleic acid compounds which comprise isolated nucleic acid sequences which encode SEQ ID NO:2 or SEQ ID NO:4. As skilled artisans will recognize, the amino acid compounds of the invention can be encoded by a multitude of different nucleic acid sequences because most of the amino acids are encoded by more than one nucleic acid triplet due to the degeneracy of the amino acid code. Because these alternative nucleic acid sequences would encode the same amino acid sequences, the present invention further comprises these alternate nucleic acid sequences.

The full length nucleic acid clones which encode the amino acid sequences given by SEQ ID NO:2 and SEQ ID NO:4, where determined using standard methods, essentially as follows. Fractions of purified beta-secretase, as isolated by the methods described in Examples 1 through 3 above, were applied to an SDS-polyacrylamide gel. Following electrophoresis, the proteins were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane and stained with coomassie blue. The stained protein bands were then excised from the membrane and placed in an automatic protein sequencer where the amino acid sequence of the protein was determined using widely known automated Edman chemistry. N-terminal fragments of the determined protein sequence, of approximately 17–20 amino acids, were then searched against protein sequence databases using commercially available BLAST methodology. A BLAST analysis of the proprietary INCYTE database (INCYTE Pharmaceuticals, Palo Alto, CA,

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94304) revealed a series of ESTs showing homology to the determined experimental sequence. The ESTs were subsequently assembled into a contiguous cDNA sequence.

5 The transcript of the assembled ESTs, however, did not contain a starting methionine. In order to elongate the 5' end of the experimental sequence 5' RACE (rapid amplification of complimentary ends) was performed. [see generally: RACE, as described in M.A. Fohrman, PCR Protocols: A Guide to Methods and Applications, McInnis et al., Eds., Academic Press, Inc, San Diego, CA, pp. 28-29 (1990); see also U.S. Patent
10 No. 5,470,722; and also see Ausubel et al, Current Protocols in Molecular Biology, Ch. 15, Eds., Wiley and Sons, N.Y. (1989-1990)] After designing suitable primers based on the information contained in the EST contiguous cDNA sequence, RACE-ready cDNA (CLONTECH Laboratories, Palo Alto, CA, 94303) was used as a template to elongate and amplify the 5' end until a
15 starting methionine was detected. PCR was then employed to generate the full length cDNA clone. E. coli were subsequently transformed with the full length cDNAs comprising SEQ ID NO:1 and SEQ ID NO:3 in order to generate sufficient copies of the nucleic acids for sequence determination.

20 The gene encoding the amyloid precursor protein protease of the present invention may also be produced using synthetic methodology, which synthesis is well known in the art. See, e.g., E.L. Brown, R. Belagaje, M.J. Ryan, and H.G. Khorana, Methods in Enzymology, 68:109-151 (1979). Additionally, the nucleic acid sequences corresponding to the gene encoding the amyloid precursor protein protease of the present invention, can also be
25 generated using conventional DNA synthesizing apparatuses such as the Applied Biosystems Model 380A or 380B DNA synthesizers. (commercially available from Applied Biosystems, Inc., 850 Lincoln Center Drive, Foster City, CA 94404) These synthesizers employ phosphoramidite chemistry. In the alternative, the more traditional phosphotriester chemistry may be

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employed to synthesize the nucleic acids of this invention. See, e.g., M.J. Gait, ed., OLIGONUCLEOTIDE SYNTHESIS, A PRACTICAL APPROACH, (1984).

The synthetic amyloid precursor protein protease gene may be designed to possess restriction endonuclease cleavage sites at either end of the transcript to facilitate isolation from and integration into expression and amplification plasmids. The restriction sites are chosen so as to properly orient the coding sequence of the target enzyme with control sequences to achieve proper in-frame reading and expression of the amyloid precursor protein protease molecule. A variety of other such cleavage sites may be incorporated depending on the particular plasmid constructs employed and may be generated by techniques well known in the art.

In an alternative methodology, the desired DNA sequences can be generated using the polymerase chain reaction as described in U.S. Patent No. 4,889,818, which is herein incorporated by reference.

In addition to the deoxyribonucleic acid compounds described supra, the present invention also encompasses ribonucleic acid compounds which have a sequence which encodes SEQ ID NO:2 or SEQ ID NO:4. Particularly, the present invention provides the RNA sequence represented by

20 GCUGUCAGC CGCCUCACAG GAAGAUGCUG CGUCGGCGGG GCAGCCCUGG 50
CAUGGGUGUG CAUGUGGGUG CAGCCCUGGG AGCACUGUGG UUCUGCCUCA 100
25 CAGGAGCCCU GGAGGUCCAG GUCCCUGAAG ACCCAGUGGU GGCACUGGUG 150
GGCACCGAUG CCACCCUGUG CUGCUCUUC UCCCUGAGC CUGGCUUCAG 200
CCUGGCACAG CUCAACCUCA UCUGGCAGCU GACAGAUACC AAACAGCUGG 250
30 UGCACAGCUU UGCUGAGGGC CAGGACCAGG GCAGCGCCUA UGCCAACCGC 300
ACGGCCCUCU UCCCGGACCU GCUGGCACAG GGCAACGCAU CCCUGAGGCU 350

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5 GCAGCGCGUG CGUGUGGCGG ACGAGGGCAG CUUCACCUGC UUCGUGAGCA 400
UCCGGGAUUU CGGCAGCGCU GCCGUCAGCC UGCAGGUGGC CGCUCCCUAC 450
UCGAAGCCCA GCAUGACCCU GGAGCCCAAC AAGGACCUGC GGCCAGGGGA 500
CACGGUGACC AUCACGUGCU CCAGCUACCA GGGCUACCCU GAGGCUGAGG 550
10 UGUUCUGGCA GGAUGGGCAG GGUGUGCCCC UGACUGGCAA CGUGACCACG 600
UCGCAGAUGG CCAACGAGCA GGGCUUGUUU GAUGUGCACA GCAUCCUGCG 650
GGUGGUGCUG GGUGCAAUG GCACCUACAG CUGCCUGGUG CGCAACCCCG 700
15 UGCUGCAGCA GGAUGCGCAC AGCUCUGUCA CCAUCACACC CCAGAGAAGC 750
CCCACAGGAG CCGUGGAGGU CCAGGUCCCU GAGGACCCGG UGGUGGCCCCU 800
20 AGUGGGCACC GAUGCCACCC UGCGCUGCUC CUUCUCCCCC GAGCCUGGCU 850
UCAGCCUGGC ACAGCUCAAC CUCAUCUGGC AGCUGACAGA CACCAAACAG 900
CUGGUGCACA GUUUCACCGA AGGCCGGGAC CAGGGCAGCG CCUAUGCCAA 1000
25 CCGCACGGCC CUCUCCCCGG ACCUGCUGGC ACAAGGCAAU GCAUCCUGA 1050
GGCUGCAGCG CGUGCGUGUG GCGGACGAGG GCAGCUUCAC CUGCUUCGUG 1100
30 AGCAUCCGGG AUUUCGGCAG CGCUGCCGUC AGCCUGCAGG UGGCCGCUCC 1150
CUACUCGAAG CCCAGCAUGA CCCUGGAGCC CAACAAGGAC CUGCGGCCAG 1200
GGGACACGGU GACCAUCACG UGUCCAGCU ACCGGGGCUA CCCUGAGGCU 1250
35 GAGGUGUUCU GGCAGGAUGG GCAGGGUGUG CCCCUGACUG GCAACGUGAC 1300
CACGUCGCAG AUGGCCAACG AGCAGGGCUU GUUUGAUGUG CACAGCGUCC 1350

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UGCGGGUGGU GCUGGGUGCG AAUGGCACCU ACAGCUGCCU GGUGCGCAAC 1400

CCCGUGCUGC AGCAGGAUGC GCACGGCUCU GUCACCAUCA CAGGGCAGCC 1450

5 UAUGACAUUC CCCCCAGAGG CCCUGUGGGU GACCGUGGGG CUGUCUGUCU 1500

GUCUCAUUGC ACUGCUGGUG GCCCUGGCUU UCGUGUGCUG GAGAAAGAUC 1550

AAACAGAGCU GUGAGGAGGA GAAUGCAGGA GCUGAGGACC AGGAUGGGGA 1560

10 GGGAGAAGGC UCCAAGACAG CCCUGCAGCC UCUGAAACAC UCUGACAGCA 1600

AAGAAGAUGA UGGACAAGAA AUAGCCUGAC CAUGAGGACC AGGGAGCUGC 1650

15 UACCCCUCCC UACAGCUCCU ACCCUCUGGC UGC 1683

which is herein referred to as SEQ ID NO:5.

The ribonucleic acids of the present invention may be prepared using the polynucleotide synthetic methods discussed supra or they may be prepared enzymatically using any one of various RNA polymerases to transcribe a DNA template. This invention also provides nucleic acids, RNA or DNA, which are complementary to SEQ ID NO:1, SEQ ID NO:3, or SEQ ID NO:5

The present invention also provides probes and primers useful for molecular biology techniques. A compound which encodes for SEQ ID NO:1, SEQ ID NO:3, or SEQ ID NO:5; or a nucleic acid complimentary to SEQ ID NO:1, SEQ ID NO:3, or SEQ ID NO:5; or a fragment thereof and which is at least 18 base pairs in length, and which will selectively hybridize to genomic DNA or messenger RNA encoding a β -secretase protease, is provided. Preferably, the 18 or more base pair compound is DNA.

Primers and probes may be obtained by means well known in the art. For example, once a protein of interest is isolated, restriction enzymes and subsequent gel separation may be used to isolate the fragment of choice.

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The term "selectively hybridize" as used herein may refer to either of two situations. In the first such embodiment of this invention, the nucleic acid compounds described supra hybridize to DNA or RNA encoding a human β -secretase protease under more stringent hybridization conditions
5 than these same nucleic acid compounds would hybridize to nucleic acids encoding analogous β -secretases of another species. In the second such embodiment of this invention, these probes hybridize to the DNA or RNA encoding the β -secretase under more stringent hybridization conditions than other related compounds, including nucleic acid sequences encoding other
10 amyloid precursor protein proteases.

These probes and primers can be prepared enzymatically as described supra. In a most preferred embodiment, however, these probes and primers are synthesized using chemical means as described herein.

Those skilled in the art will recognize the techniques associated
15 with probes and primers are well known. For example, all or part of the probes or primers may be used to hybridize to the coding sequence. Then, through PCR amplification, the full length sequence may be generated. The full length sequence can be subsequently subcloned into any vector of choice. Alternatively, the primers or probes may be radioactively labeled at the 5' end
20 in order to screen cDNA libraries by conventional means. A primer or probe can be labeled with a radioactive element which provides for an adequate signal as a means for detection and has sufficient half-life to be useful for detection, such as ^{32}P , ^3H , ^{14}C or the like. Other materials which can be used to label the primer or probe include antigens that are recognized by a
25 specific labeled antibody, fluorescent compounds, enzymes, antibodies specific for a labeled antigen, and chemiluminescent compounds. An appropriate label can be selected having regard to the rate of hybridization and binding of the primer or probe to the nucleotide to be detected and the amount of nucleotide available for hybridization.

30 As discussed supra, recombinant DNA cloning vectors and

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expression vectors comprising the nucleic acids of the present invention can be prepared. Many such vectors are illustrated and described above. The preferred nucleic acid vectors are those which are DNA. The preferred recombinant DNA vectors comprise the isolated DNA sequences as given by
5 SEQ ID NO:1 or SEQ ID NO:3. The most preferred recombinant DNA vectors comprise the DNA sequence given by SEQ ID NO:3.

The skilled artisan understands that the type of cloning vector employed depends upon the availability of appropriate restriction sites, the type of host cell in which the vector is to be transfected or transformed, the
10 purpose of the transfection or transformation (e.g., transient expression in an oocyte system, stable transformation as an extrachromosomal element, or integration into the host chromosome), the presence or absence of readily assayable markers (e.g., antibiotic resistance markers, metabolic markers, or the like), and the number of copies of the gene to be present in the cell.

15 The type of vector employed to carry the nucleic acids of the present invention may be RNA viruses, DNA viruses, lytic bacteriophages, lysogenic bacteriophages, stable bacteriophages, plasmids, viroids, and the like. When preparing an expression vector, the skilled artisan understands that there are many variables to be considered. One such example is the use
20 of a constitutive promoter, i.e. a promoter which is functional at all times, instead of a regulatable promoter which may be activated or inactivated by the artisan using heat, addition or removal of a nutrient, addition of an antibiotic, and the like. The practitioner also understands that the amount of the nucleic acid or protein to be produced dictates, in part, the selection of the
25 expression system. For experiments examining the amount of the protein expressed on the cell membrane or for experiments examining the biological function of an expressed membrane protein, for example, it may be unwise to employ an expression system which produces too much of the protein. The addition or subtraction of certain sequences, such as a signal sequence
30 preceding the coding sequence, may be employed by the practitioner to

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influence localization of the resulting polypeptide. Such sequences added to or removed from the nucleic acid compounds of the present invention are encompassed within this invention.

Plasmids can be readily modified to construct expression vectors
5 in a variety of organisms, including, but not limited to, E. coli, Sf9 (as host for baculovirus), Spodoptera and Saccharomyces. See: Serook et al., Molecular Cloning: A Laboratory Manual (1989)

One of the most widely employed techniques for altering a nucleic acid sequence is by way of oligonucleotide-directed site-specific
10 mutagenesis. B Comack, "Current Protocols in Molecular Biology", 8.01-8.5.9, (F. Ausubel, et al., eds. 1991). In this technique an oligonucleotide, whose sequence contains the mutation of interest, is synthesized as described supra. This oligonucleotide is then hybridized to a template containing the wild-type sequence. In a most preferred embodiment of this technique, the template is
15 a single-stranded template. Particularly useful are plasmids which contain regions such as the fl intergenic region. This region allows the generation of single-stranded templates when a helper phage is added to the culture harboring the "phagemid".

After the annealing of the oligonucleotide, to the template, a
20 DNA-dependent DNA polymerase is then used to synthesize the second strand from the oligonucleotide, complementary to the template DNA. The resulting product is a heteroduplex molecule containing a mismatch due to the mutation in the oligonucleotide. After DNA replication by the host cell a mixture of two types of plasmid are present, the wild-type and the newly
25 constructed mutant. This technique permits the introduction of convenient restriction sites such that the coding sequence may be placed immediately adjacent to whichever transcriptional or translational regulatory elements are employed by the practitioner.

The construction protocols utilized for E. coli can be followed to
30 construct analogous vectors for other organisms, merely by substituting, if

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necessary, the appropriate regulatory elements using techniques well known to skilled artisans.

Host cells which harbor the nucleic acids provided by the present invention can also be prepared. One suitable host cell is an Xenopus sp. oocyte which has been injected with RNA or DNA compounds of the present invention. Preferred oocytes of the present invention are those which harbor a sense mRNA of the present invention. Other preferred host cells include HeLa and 293 cells which have been transfected and/or transformed with a vector which comprises a nucleic acid of the present invention.

A method for constructing a recombinant host cell capable of expressing SEQ ID NO:2 or SEQ ID NO:4 is also possible with regard to the present invention, said method comprising transforming a host cell with a recombinant DNA vector that comprises an isolated DNA sequence which encodes SEQ ID NO:2 or SEQ ID NO:4. The preferred host cell is 293 cells. A preferred vector for expression is one which comprises SEQ ID NO:1 or SEQ ID NO:3, most preferably SEQ ID NO:1. Transformed host cells may be cultured under conditions well known to skilled artisans such that SEQ ID NO: 2 or SEQ ID NO:4 are expressed, thereby producing the sequence of β -secretase in the recombinant host cell.

A further embodiment of the invention consists of a method of isolating and purifying an amyloid precursor protein protease from a host cell expressing said protein. In this embodiment, a host cell, either prokaryotic or eukaryotic, expressing amyloid precursor protein protease, is cultured in an appropriate medium until a substantial cell mass has been obtained. The second step of this embodiment is the isolation of amyloid precursor protein protease from the cultured cells. Two methods for purifying amyloid precursor protein protease from a non-transformed mammalian cell line are described in United States Patent Number 5,328,842, the entire contents of

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which are herein incorporated by reference. The following summarizes those methods.

Once grown and harvested, the cultured cells are lysed by nitrogen cavitation in the presence of protease inhibitors. A soluble fraction
5 is prepared from the lysate by ultracentrifugation. The resulting solution of cytosolic proteins contains β -secretase and is subjected to a series of purification procedures.

The soluble fraction of the cell lysate is run through a series of column chromatography procedures. Anion exchange chromatography is
10 followed by hydrophobic interaction, molecular sizing, and finally another hydrophobic interaction technique where the conditions are such that the β -secretase binds the resin weakly. Each column is run individually, and the eluate is collected in fractions while monitoring for absorbance at 280 nm. Fractions are assayed for β -secretase activity, and those fractions with the
15 desired activity are then run over the next column until a homogeneous solution of β -secretase is obtained.

Immunoaffinity purification using anti- β -secretase antibodies is an alternative to the series of chromatographic procedures already mentioned. Making antiserum or monoclonal antibodies directed against a
20 purified protein is well known in the art, and skilled artisans readily will be able to prepare anti- β -secretase antibodies. Preparing an immunoaffinity matrix using such antibodies and isolating β -secretase using the immunoaffinity matrix is also well within the skill of the art. See, AFFINITY CHROMATOGRAPHY PRINCIPLES & METHODS, Pharmacia Fine Chemicals, 1983.

25 The ability of an agent to inhibit the protein of the present invention is essential in the development of a multitude of indications. In developing agents which act as inhibitors of β -secretase, it would be desirable, therefore, to determine those agents which interact with the protein of the present invention. Generally, such an assay includes a method for
30 determining whether a substance is a functional ligand of β -secretase, said

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method comprising contacting a functional compound of the β -secretase with said substance, monitoring enzymatic activity by physically detectable means, and identifying those substances which effect a chosen response.

The instant invention provides such a screening system useful
5 for discovering agents which inhibit the β -secretase, said screening system comprising the steps of:

- a) isolating a β -secretase;
- b) exposing said β -secretase to a potential inhibitor of the β -secretase;
- 10 c) introducing a suitable substrate;
- d) quantifying the amount of cleavage of the substrate relative to a control in which no potential inhibitor has been introduced.

This allows one to rapidly screen for inhibitors of β -secretase.
15 Utilization of the screening system described above provides a sensitive and rapid means to determine compounds which inhibit β -secretase. This screening system may also be adapted to automated procedures such as a PANDEX® (Baxter-Dade Diagnostics) system allowing for efficient high-volume screening of potential therapeutic agents. In such a screening
20 protocol a protein protease is prepared as elsewhere described herein, preferably using recombinant DNA technology. A sample of a test compound is then introduced to the reaction vessel containing the protein protease followed by the addition of an appropriate substrate. In the alternative the substrate may be added simultaneously with the test compound.

25 The desirability of a bioactivity assay system which determines the response of β -secretase to a compound of interest is clear. The instant invention provides such a bioactivity assay, said assay comprising the steps of:

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- a) transfecting a mammalian host cell with an expression vector comprising DNA encoding β -secretase;
- 5 b) culturing said host cell under conditions such that the β -secretase protein is expressed;
- c) exposing said host cell so transfected to a test compound; and
- 10 d) measuring the change in a physiological condition known to be influenced by β -secretase relative to a control in which the transfected host cell is not exposed to a test compound.

The present invention comprises a method of using said

15 inhibitors of β -secretase in the treatment of patients with acquired disease states of the brain including Alzheimer's disease. As used herein, "patient" refers to a mammal such a rat, mouse, guinea pig, dog, or human. It is understood, however, that the preferred patient for use with the methods herein is a human patient. As used herein, the term "treatment" (or "treat"

20 or "treating") includes its generally accepted meaning which encompasses prohibiting, preventing, restraining, and slowing, stopping, or reversing the progression, severity, and resulting symptoms of acquired disease states of the brain. As such, the methods of the present invention encompass both therapeutic and prophylactic uses.

25 Compounds which are inhibitors of β -secretase are usually administered in the form of pharmaceutical compositions. These compounds can be administered by a variety of routes including oral, rectal, transdermal, subcutaneous, intravenous, intramuscular, and intranasal. These compounds are effective as both injectable and oral compositions. Such compositions are

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prepared in a manner well known in the pharmaceutical art and comprise at least one active compound.

The present invention also includes methods employing pharmaceutical compositions which contain, as the active ingredient, inhibitors of β -secretase associated with pharmaceutically acceptable carriers. In making the compositions of the present invention the active ingredient is usually mixed with an excipient, diluted by an excipient or enclosed within such a carrier which can be in the form of a capsule, sachet, paper or other container. When the excipient serves as a diluent, it can be a solid, semi-
10 solid, or liquid material, which acts as a vehicle, carrier or medium for the active ingredient. Thus, the compositions can be in the form of tablets, pills, powders, lozenges, sachets, cachets, elixirs, suspensions, emulsions, solutions, syrups, aerosols (as a solid or in a liquid medium), ointments containing for example up to 10% by weight of the active compound, soft and hard gelatin
15 capsules, suppositories, sterile injectable solutions, and sterile packaged powders.

In preparing a formulation, it may be necessary to mill the active compound to provide the appropriate particle size prior to combining with the other ingredients. If the active compound is substantially insoluble,
20 it ordinarily is milled to a particle size of less than 200 mesh. If the active compound is substantially water soluble, the particle size is normally adjusted by milling to provide a substantially uniform distribution in the formulation, e.g. about 40 mesh.

Some examples of suitable excipients include lactose, dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, calcium phosphate, alginates, tragacanth, gelatin, calcium silicate, microcrystalline cellulose, polyvinylpyrrolidone, cellulose, water, syrup, and methyl cellulose. The formulations can additionally include: lubricating agents such as talc, magnesium stearate, and mineral oil; wetting agents; emulsifying and
30 suspending agents; preserving agents such as methyl- and

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propylhydroxybenzoates; sweetening agents; and flavoring agents. The compositions of the invention can be formulated so as to provide quick, sustained or delayed release of the active ingredient after administration to the patient by employing procedures known in the art.

5 The compositions are preferably formulated in a unit dosage form, each dosage containing from about 5 to about 100 mg, more usually about 10 to about 30 mg, of the active ingredient. The term "unit dosage form" refers to physically discrete units suitable as unitary dosages for human subjects and other mammals, each unit containing a predetermined
10 quantity of active material calculated to produce the desired therapeutic effect, in association with a suitable pharmaceutical excipient.

 The active compound is effective over a wide dosage range. For examples, dosages per day normally fall within the range of about 0.5 to about 30 mg/kg of body weight. In the treatment of adult humans, the range
15 of about 1 to about 15 mg/kg/day, in single or divided dose, is especially preferred. However, it will be understood that the amount of the compound actually administered will be determined by a physician, in the light of the relevant circumstances, including the condition to be treated, the chosen route of administration, the actual compound administered, the age, weight,
20 and response of the individual patient, and the severity of the patient's symptoms, and therefore the above dosage ranges are not intended to limit the scope of the invention in any way. In some instances dosage levels below the lower limit of the aforesaid range may be more than adequate, while in other cases still larger doses may be employed without causing any harmful
25 side effect, provided that such larger doses are first divided into several smaller doses for administration throughout the day.

 For preparing solid compositions such as tablets the principal active ingredient is mixed with a pharmaceutical excipient to form a solid preformulation composition containing a homogeneous mixture of a
30 compound of the present invention. When referring to these preformulation

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compositions as homogeneous, it is meant that the active ingredient is dispersed evenly throughout the composition so that the composition may be readily subdivided into equally effective unit dosage forms such as tablets, pills and capsules. This solid preformulation is then subdivided into unit
5 dosage forms of the type described above containing from 0.1 to about 500 mg of the active ingredient of the present invention.

The tablets or pills of the present invention may be coated or otherwise compounded to provide a dosage form affording the advantage of prolonged action. For example, the tablet or pill can comprise an inner
10 dosage and an outer dosage component, the latter being in the form of an envelope over the former. The two components can be separated by enteric layer which serves to resist disintegration in the stomach and permit the inner component to pass intact into the duodenum or to be delayed in release. A variety of materials can be used for such enteric layers or coatings, such
15 materials including a number of polymeric acids and mixtures of polymeric acids with such materials as shellac, cetyl alcohol, and cellulose acetate.

The liquid forms in which the novel compositions of the present invention may be incorporated for administration orally or by injection include aqueous solutions, suitably flavored syrups, aqueous or oil
20 suspensions, and flavored emulsions with edible oils such as cottonseed oil, sesame oil, coconut oil, or peanut oil, as well as elixirs and similar pharmaceutical vehicles.

Compositions for inhalation or insufflation include solutions and suspensions in pharmaceutically acceptable, aqueous or organic solvents, or
25 mixtures thereof, and powders. The liquid or solid compositions may contain suitable pharmaceutically acceptable excipients as described supra. Preferably the compositions are administered by the oral or nasal respiratory route for local or systemic effect. Compositions in preferably pharmaceutically acceptable solvents may be nebulized by use of inert gases.
30 Nebulized solutions may be breathed directly from the nebulizing device or

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the nebulizing device may be attached to a face mask, tent, or intermittent positive pressure breathing machine. Solution, suspension, or powder compositions may be administered, preferably orally or nasally, from devices which deliver the formulation in an appropriate manner.

- 5 The following examples illustrate the pharmaceutical compositions of the present invention.

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Formulation Preparation 1

Hard gelatin capsules containing the following ingredients are prepared:

5

<u>Ingredient</u>		<u>Quantity</u>
Active Ingredient		<u>(mg/capsule)</u>
		30.0
10	Starch	305.0
	Magnesium stearate	5.0

The above ingredients are mixed and filled into hard gelatin capsules in 340 mg quantities.

15

Formulation Preparation 2

A tablet formula is prepared using the ingredients below:

20

<u>Ingredient</u>		<u>Quantity</u>
Active Ingredient		<u>(mg/tablet)</u>
		25.0
25	Cellulose, microcrystalline	200.0
	Colloidal silicon dioxide	10.0
	Stearic acid	5.0

30

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The components are blended and compressed to form tablets, each weighing 240 mg.

Formulation Preparation 3

5

A dry powder inhaler formulation is prepared containing the following components:

	<u>Ingredient</u>	<u>Weight %</u>
10	Active Ingredient	5
	Lactose	95

The active mixture is mixed with the lactose and the mixture is added to a dry powder inhaling appliance.

15

Formulation Preparation 4

Tablets, each containing 30 mg of active ingredient, are prepared as follows:

20

	<u>Ingredient</u>	<u>Quantity (mg/tablet)</u>
	Active Ingredient	30.0 mg
25	Starch	45.0 mg
	Microcrystalline cellulose	35.0 mg
	Polyvinylpyrrolidone	
30	(as 10% solution in water)	4.0 mg

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	Sodium carboxymethyl starch	4.5 mg
	Magnesium stearate	0.5 mg
5	Talc	<u>1.0 mg</u>
	Total	120 mg

10 The active ingredient, starch and cellulose are passed through a No. 20 mesh U.S. sieve and mixed thoroughly. The solution of polyvinylpyrrolidone is mixed with the resultant powders, which are then passed through a 16 mesh U.S. sieve. The granules so produced are dried at 50-60°C and passed through a 16 mesh U.S. sieve. The sodium

15 carboxymethyl starch, magnesium stearate, and talc, previously passed through a No. 30 mesh U.S. sieve, are then added to the granules which, after mixing, are compressed on a tablet machine to yield tablets each weighing 120 mg.

20 Formulation Preparation 5

Capsules, each containing 40 mg of medicament are made as follows:

25		Quantity
	<u>Ingredient</u>	<u>(mg/capsule)</u>
	Active Ingredient	40.0 mg
	Starch	109.0 mg
30		

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Magnesium stearate	<u>1.0 mg</u>
--------------------	---------------

Total	150.0 mg
-------	----------

- 5 The active ingredient, cellulose, starch, and magnesium stearate are blended, passed through a No. 20 mesh U.S. sieve, and filled into hard gelatin capsules in 150 mg quantities.

Formulation Preparation 6

10

Suppositories, each containing 25 mg of active ingredient are made as follows:

	<u>Ingredient</u>	<u>Amount</u>
15	Active Ingredient	25 mg
	Saturated fatty acid glycerides to	2,000 mg

- 20 The active ingredient is passed through a No. 60 mesh U.S. sieve and suspended in the saturated fatty acid glycerides previously melted using the minimum heat necessary. The mixture is then poured into a suppository mold of nominal 2.0 g capacity and allowed to cool.

Formulation Preparation 7

25

Suspensions, each containing 50 mg of medicament per 5.0 ml dose are made as follows:

	<u>Ingredient</u>	<u>Amount</u>
30	Active Ingredient	50.0 mg

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	Xanthan gum	4.0 mg
	Sodium carboxymethyl cellulose (11%)	
5	Microcrystalline cellulose (89%)	50.0 mg
	Sucrose	1.75 g
	Sodium benzoate	10.0 mg
10	Flavor and Color	q.v.
	Purified water to	5.0 ml

15 The medicament, sucrose and xanthan gum are blended, passed
through a No. 10 mesh U.S. sieve, and then mixed with a previously made
solution of the microcrystalline cellulose and sodium carboxymethyl cellulose
in water. The sodium benzoate, flavor, and color are diluted with some of the
water and added with stirring. Sufficient water is then added to produce the
20 required volume.

Formulation Preparation 8

 Capsules, each containing 15 mg of medicament, are made as
25 follows:

	<u>Ingredient</u>	<u>Quantity</u>
		<u>(mg/capsule)</u>
	Active Ingredient	15.0 mg
30		

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	Starch	407.0 mg
	Magnesium stearate	<u>3.0 mg</u>
5	Total	425.0 mg

The active ingredient, cellulose, starch, and magnesium stearate are blended, passed through a No. 20 mesh U.S. sieve, and filled into hard gelatin capsules in 425 mg quantities.

10

Formulation Preparation 9

An intravenous formulation may be prepared as follows:

15

<u>Ingredient</u>	<u>Quantity</u>
Active Ingredient	250.0 mg
Isotonic saline	1000 ml

20

Formulation Preparation 10

A topical formulation may be prepared as follows:

25	<u>Ingredient</u>	<u>Quantity</u>
	Active Ingredient	1-10 g
	Emulsifying Wax	30 g
30	Liquid Paraffin	20 g

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White Soft Paraffin

to 100 g

5 The white soft paraffin is heated until molten. The liquid paraffin and emulsifying wax are incorporated and stirred until dissolved. The active ingredient is added and stirring is continued until dispersed. The mixture is then cooled until solid.

Formulation Preparation 11

10

Sublingual or buccal tablets, each containing 10 mg of active ingredient, may be prepared as follows:

15	<u>Ingredient</u>	<u>Quantity</u> <u>Per Tablet</u>
	Active Ingredient(s)	10.0 mg
	Glycerol	210.5 mg
20	Water	143.0 mg
	Sodium Citrate	4.5 mg
	Polyvinyl Alcohol	26.5 mg
25	Polyvinylpyrrolidone	<u>15.5 mg</u>
	Total	410.0 mg

30 The glycerol, water, sodium citrate, polyvinyl alcohol, and polyvinylpyrrolidone are admixed together by continuous stirring and

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maintaining the temperature at about 90°C. When the polymers have gone into solution, the solution is cooled to about 50-55°C and the medicament is slowly admixed. The homogenous mixture is poured into forms made of an inert material to produce a drug-containing diffusion matrix having a
5 thickness of about 2-4 mm. This diffusion matrix is then cut to form individual tablets having the appropriate size.

Another preferred formulation employed in the methods of the present invention employs transdermal delivery devices ("patches"). Such transdermal patches may be used to provide continuous or discontinuous
10 infusion of the compounds of the present invention in controlled amounts. The construction and use of transdermal patches for the delivery of pharmaceutical agents is well known in the art. See, e.g., U.S. Patent 5,023,252, issued June 11, 1991, herein incorporated by reference. Such patches may be constructed for continuous, pulsatile, or on demand delivery
15 of pharmaceutical agents.

Frequently, it will be desirable or necessary to introduce the pharmaceutical composition to the brain, either directly or indirectly. Direct techniques usually involve placement of a drug delivery catheter into the host's ventricular system to bypass the blood-brain barrier. One such
20 implantable delivery system, used for the transport of biological factors to specific anatomical regions of the body, is described in U.S. Patent 5,011,472, issued April 30, 1991, which is herein incorporated by reference.

Indirect techniques, which are generally preferred, usually involve formulating the compositions to provide for drug latentiation by the
25 conversion of hydrophilic drugs into lipid-soluble drugs or prodrugs. Latentiation is generally achieved through blocking of the hydroxy, carbonyl, sulfate, and primary amine groups present on the drug to render the drug more lipid soluble and amenable to transportation across the blood-brain barrier. Alternatively, the delivery of hydrophilic drugs may be enhanced by

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intra-arterial infusion of hypertonic solutions which can transiently open the blood-brain barrier.

The proteins of this invention as well as fragments of these proteins may be used as antigens for the synthesis of antibodies. The term
5 “antibody” as used herein describes antibodies, fragments of antibodies (such as, but not limited, to Fab, Fab', Fab₂', and Fv fragments), and chimeric, humanized, veneered, resurfaced, or CDR-grafted antibodies capable of binding antigens of a similar nature as the parent antibody molecule from which they are derived. The instant invention also encompasses single chain
10 polypeptide binding molecules.

The term “antibody” as used herein is not limited by the manner in which the antibodies are produced, whether such production is in situ or not. The term “antibody” as used in this specification encompasses those antibodies produced by recombinant DNA technology means including, but
15 not limited, to expression in bacteria, yeast, insect cell lines, or mammalian cell lines.

The production of antibodies, both monoclonal and polyclonal, in animals, especially mice, is well known in the art. See, e.g., C. Milstein, HANDBOOK OF EXPERIMENTAL IMMUNOLOGY, (Blackwell Scientific Pub., 1986);
20 J. Goding, MONOCLONAL ANTIBODIES: PRINCIPLES AND PRACTICE, (Academic Press, 1983). For the production of monoclonal antibodies the basic process begins with injecting a mouse, or other suitable animal, with an immunogen. The mouse is subsequently sacrificed and cells taken from its spleen are fused with myeloma cells, resulting in a hybridoma that reproduces in vitro. The
25 population of hybridomas is screened to isolate individual clones, each of which secretes a single antibody species, specific for the immunogen. The individual antibody species obtained in this way is each the product of a single B cell from the immune animal generated in response to a specific antigenic site, or epitope, recognized on the immunogenic substance.

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Chimeric antibodies are described in U.S. Patent No. 4,816,567, which issued March 28, 1989 to S. Cabilly, et al. This reference discloses methods and vectors for the preparation of chimeric antibodies. The entire contents of U.S. Patent No. 4,816,567 are herein incorporated by reference.

5 An alternative approach to production of genetically engineered antibodies is provided in U.S. Patent No. 4,816,397, which also issued March 28, 1989 to M. Boss, et al., the entire contents of which are herein incorporated by reference. The Boss patent teaches the simultaneous co-expression of the heavy and light chains of the antibody in the same host cell.

10 The approach of U.S. Patent 4,816,397 has been further refined as taught in European Patent Publication No. 0 239 400, which published September 30, 1987. The teachings of this European patent publication (Winter) are a preferred format for the genetic engineering of the reactive monoclonal antibodies of this invention. The Winter technology involves the
15 replacement of complementarity determining regions (CDRs) of a human antibody with the CDRs of a murine monoclonal antibody thereby converting the specificity of the human antibody to the specificity of the murine antibody which was the source of the CDR regions. This "CDR grafting" technology affords a molecule containing minimal murine sequence and thus is less
20 immunogenic.

Single chain antibody technology is yet another variety of genetically engineered antibody which is now well known in the art. See, e.g. R.E. Bird, et al., Science 242:423-426 (1988); PCT Publication No. WO 88/01649, which was published 10 March 1988; United States Patent
25 5,260,203, issued November 9, 1993, the entire contents of which are herein incorporated by reference. The single chain antibody technology involves joining the binding regions of heavy and light chains with a polypeptide sequence to generate a single polypeptide having the binding specificity of the antibody from which it was derived.

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The aforementioned genetic engineering approaches provide the skilled artisan with numerous means to generate molecules which retain the binding characteristics of the parental antibody while affording a less immunogenic format.

5 These antibodies are used in diagnostics, therapeutics or in diagnostic/therapeutic combinations. By "diagnostics" as used herein is meant testing that is related to either the in vitro or in vivo diagnosis of disease states or biological status in mammals, preferably in humans. By "therapeutics" and "therapeutic/diagnostic combinations" as used herein is
10 respectively meant the treatment or the diagnosis and treatment of disease states or biological status by the in vivo administration to mammals, preferably humans, of the antibodies of the present invention. The antibodies of the present invention are especially preferred in the diagnosis and/or treatment of conditions associated with an excess or deficiency of β -secretase.

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5 WHAT IS CLAIMED IS:

1. An isolated amino acid compound functional as an amyloid precursor protein protease which comprises the amino acid sequence given by SEQ ID NO:2 or SEQ ID NO:4, or a functional equivalent thereof, or a
10 fragment of at least 6 continuous amino acids thereof.
2. A nucleic acid compound encoding the amino acid compound of Claim 1.
- 15 3. A nucleic acid compound encoding the amino acid compound of Claim 1 which comprises the nucleic acid sequence given by SEQ ID NO:1 or SEQ ID NO:3.
4. A composition comprising an isolated nucleic acid compound
20 containing a sequence encoding an amyloid precursor protein protease as claimed in Claim 2, wherein said sequence is selected from the group consisting of
 - (a) SEQ ID NO:1 or SEQ ID NO:3;
 - (b) nucleotides 25 through 1629 of SEQ ID NO:1
25 or SEQ ID NO: 3;
 - (c) SEQ ID NO:5;
 - (d) nucleotides 25 through 1629 of SEQ ID NO:5;
 - (e) a nucleic acid complimentary to (a), (b), (c), or (d); and

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- (f) a fragment of (a), (b), (c), (d), or (e) that is at least 18 bases in length and which will selectively hybridize to human genomic DNA encoding an amyloid precursor protein protease.

5

5. The composition as claimed in Claim 4 wherein the isolated nucleic acid is deoxyribonucleic acid.

6. The composition as claimed in Claim 5 wherein the
10 deoxyribonucleic acid is SEQ ID NO:1 or SEQ ID NO:3, or a sequence complimentary to SEQ ID NO:1 or SEQ ID NO:3.

7. The composition of Claim 5 wherein the deoxyribonucleic acid
is nucleotides 25 through 1629 of SEQ ID NO:1 or SEQ ID NO:3, or a
15 sequence complimentary to nucleotides 25 through 1629 of SEQ ID NO:1.

8. The composition as claimed in Claim 4 wherein the isolated nucleic acid is ribonucleic acid.

20 9. The composition as claimed in Claim 8 wherein the ribonucleic acid is SEQ ID NO:5 or a sequence complimentary to SEQ ID NO:5.

10. The composition of Claim 8 wherein the ribonucleic acid is
25 nucleotides 25 through 1629 of SEQ ID NO:5 or a sequence complimentary to nucleotides 25 through 1629 of SEQ ID NO:5.

11. An expression vector capable of producing an amyloid precursor protein protease in a host cell which comprises a nucleic acid

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compound as claimed in Claim 4 in combination with regulatory elements necessary for expresison of the nucleic acid compound in a host cell.

12. An expression vector as claimed in Claim 11 for use in a host
5 cell wherein said host cell is a mammalian cell.

13. An expression vector as claimed in Claim 11 for use in a host cell wherein said host cell is a prokaryotic cell.

14. A transfected host cell harboring an expression vector as
10 claimed in Claim 11.

15. A transfected host cell as claimed in Claim 14 wherein the
host cell is a mammalian cell.

15

16. A transfected host cell as claimed in Claim 14 wherein the
host cell is a prokaryotic cell.

17. A method to identify compounds which inhibit the synthesis
20 or release of beta-amyloid protien, which method comprises:

(a) isolating a β -secretase;

(b) exposing said β -secretase to a potential inhibitor of the β -secretase;

(c) introducing a suitable substrate;

25 (d) quantifying the amount of cleavage of the substrate relative to a control in which no potential inhibitor has been introduced.

18. The method of Claim 17 wherein said β -secretase comprises the amino acid sequence given by SEQ ID NO:2 or SEQ ID NO:4.

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19. A method of evaluating the effectiveness of a test compound for the treatment or prevention of a condition associated with the synthesis or release of beta-amyloid protein, which method comprises:

- 5 (a) transfecting a mammalian host cell with an expression vector comprising DNA encoding β -secretase;
- (b) culturing said host cell under conditions such that the β -secretase protein is expressed;
- (c) exposing said host cell so transfected to a test compound; and
- 10 (d) measuring the change in a physiological condition known to be influenced by β -secretase relative to a control in which the transfected host cell is not exposed to a test compound.

20. The method of Claim 19 wherein said β -secretase comprises the amino acid sequence given by SEQ ID NO:2 or SEQ ID NO:4.

- 15 21. A method of treating or preventing Alzheimer's Disease or other neurodegenerative disorders in a patient which comprises administering to a patient in need thereof, an effective amount of an inhibitor of the amyloid precursor protein protease given by the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:4.

- 20 22. A pharmaceutical composition comprising an inhibitor of the amyloid precursor protein protease given by SEQ ID NO:2 or SEQ ID NO:4, in combination with one or more pharmaceutically acceptable excipients, carriers, or diluents therefor.

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/06707

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07K14/47 C12N15/57 C12N9/64 C12N5/10 C12N5/16
A61P25/28

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C07K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

STRAND, EMBL, GENSEQ, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE GENEMBL 'Online! 6 June 1996 (1996-06-06) HILLIER ET AL.: "yx480g8.r1 soares melanocyte 2NbHM Homo sapiens cDNA clone IMAGE:265022 5' similar to contains Alu repetitive element" XP002148339 Accession number N30496	1-16
X	WO 96 40885 A (MCCONLOGUE LISA C ;KEIM PAMELA S (US); SINHA SUKANTO (US); TAN HUA) 19 December 1996 (1996-12-19) page 22, line 20 -page 23, line 2 page 41, line 20 -page 48, line 34 claims 23-41; table 3 -/-	17

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

4 October 2000

Date of mailing of the international search report

17/10/2000

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/06707

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 653 154 A (HOECHST JAPAN) 17 May 1995 (1995-05-17) page 5, line 13-21 claim 10	19
A	_____ DATABASE SWISSPROT 'Online! 1 June 1998 (1998-06-01) HUSAINI ET AL.: "Bos taurus butyrophilin fragment" XP002148340 Accession No. 046535	1-16
P, X	_____ WO 99 46281 A (BAKER KEVIN P ;CHEN JIAN (US); GENENTECH INC (US); GURNEY AUSTIN () 16 September 1999 (1999-09-16) page 12, line 24-35 page 60, line 10-29 page 128, line 15-22 page 149, line 30-33 page 151, line 22-30 page 159, line 17-19 _____	1-16

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 21,22

Claims 21 and 22 refer to an antagonist/inhibitor of the polypeptide without giving a true technical characterization. Moreover, no such compounds are defined in the application. In consequence, the scope of said claims is ambiguous and vague, and their subject-matter is not sufficiently disclosed and supported (Art. 6 PCT). No search could be carried out for such purely speculative claims whose wording is, in fact, a mere recitation of the result to be achieved.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 00/06707

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